Characterization of new natural products from fungi of the German Wadden Sea

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ZUSAMMENFASSUNG

Pilze sind hervorragende Produzenten von Naturstoffen, die für pharmazeutische und agrarwirtschaftliche Anwendungen genutzt werden. Das Ziel der vorliegenden Doktorarbeit war es, dieses außergewöhnliche Biosynthesepotential für die Suche nach neuen Naturstoffen in Pilzisolaten aus dem deutschen Wattenmeer auszuschöpfen. Um eine effiziente und zielgerichtete Screening-Strategie zu entwickeln, wurden vier Kriterien zur Auswahl derjenigen Pilze herangezogen, die anschließend umfassend auf ihre Fähigkeiten zur Biosynthese von Naturstoffen hin untersucht wurden: (i) Pilzisolate von wenig erforschten Ökosystemen, (ii) weniger intensiv untersuchte Gattungen, (iii) genetisches Potential anhand des Sequenznachweises für Polyketidsynthase- und nichtribosomale Peptidsynthetase-Gene, (iv) stimulierende Effekte auf die Biosynthese durch die Variation von Kulturbedingungen. Die Screening-Strategie zahlte sich aus, denn es wurden neun neue Naturstoffe, die Calcaripeptide A–C, Calcaride A–E und ein Tropolon-Derivat, entdeckt.

Die Calcaripeptide sind Cyclodepsipeptide, die von einem *Calcarisporium* sp. (Stamm KF525) isoliert wurden. Die Strukturaufklärung mit Hilfe von ein- und zweidimensionaler Kernspinresonanzspektroskopie wies einen ungewöhnlichen Makrocyclus bestehend aus zwei Aminosäuren und eine nicht-peptidische Teilstruktur für die Substanzen nach. Die absolute Konfiguration der Calcaripeptide wurde über Röntgenstrukturanalysen in Kombination mit der erweiterten Marfey-Methode bestimmt. Biosynthesestudien zu Calcaripeptid A zeigten, dass der nicht-peptidische Strukturteil von Acetat und L-Methionin stammt, was einen biosynthetischen Ursprung von einem Hybrid aus einer Polyketidsynthase und einer nichtribosomalen Peptidsynthetase nahelegt. Trotz breit angelegter Tests zum Nachweis biologischer Aktivitäten wiesen die Calcaripeptide keine inhibitorischen Eigenschaften auf.

Zusätzlich produzierte der Stamm KF525 eine zweite Klasse von Naturstoffen, welche die neuen Calcaride A–E und fünf strukturverwandte Substanzen vom 15G256-Typ umfasste. Bei den Substanzen handelt es sich um lineare und makrocyclische Polyester, wobei die Calcaride methylierte Formen der Substanzen vom 15G256-Typ darstellen. Die absolute Konfiguration der Metabolite wurde über Messungen der optischen Rotation untersucht. Die Calcaride und Substanzen vom 15G256-Typ zeigten antibakterielle Aktivitäten gegenüber *Staphylococcus epidermidis*, *Xanthomonas*

campestris und *Propionibacterium acnes*, welche einer engen Struktur-Aktivitäts-Beziehung unterlagen.

Von einem anderen Pilzstamm, *Cladosporium* sp. KF501, wurden Mitglieder der Familie pilzlicher Tropolone isoliert. Unter den Substanzen befand sich ein neues Derivat, das ein Stereoisomer der bekannten Tropolone des Pilzstamms darstellt. Die relative Konfiguration des neuen Derivats wurde anhand einer Röntgenstrukturanalyse an einem Einkristall bestimmt. Die isolierten Tropolone wiesen antibakterielle und antifungale Eigenschaften in Testungen mit *Xanthomonas campestris* und *Trichophyton rubrum* auf.

Insgesamt demonstrieren die Ergebnisse der Arbeit den hohen Wert, den Pilze aus marinen Habitaten für die Entdeckung neuer bioaktiver Naturstoffe besitzen.

SUMMARY

Fungi are prolific producers of natural products used for pharmaceutical and agricultural applications. The scope of the present PhD study was to exploit this extraordinary biosynthetic potential for the search for new natural products from fungal isolates of the German Wadden Sea. In order to create a smart screening strategy, four criteria were applied to select fungi for subsequent thorough investigation with regard to their natural product biosynthetic abilities: (i) fungal isolates from a little-explored ecosystem, (ii) less studied genera, (iii) genetic potential according to polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) gene sequences, (iv) stimulating effects on biosynthesis by variation of culture conditions. The screening strategy was proven effective as it led to the discovery of nine new natural products, namely, calcaripeptides A–C, calcarides A–E and one tropolone derivative.

The calcaripeptides are cyclodepsipeptides that were isolated from a *Calcarisporium* sp. (strain KF525). The structure elucidation employing one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy techniques proved the compounds to possess an unusual macrocycle composed of two amino acids and a nonpeptidic substructure. The absolute configuration of the calcaripeptides was determined by X-ray crystallography in combination with the advanced Marfey's method. Biosynthetic studies on calcaripeptide A showed that the nonpeptidic structural part of the compound originated from acetate and L-methionine, suggesting a biosynthetic origin from a PKS–NRPS hybrid. Despite broad testing for biological activities, the calcaripeptides did not exhibit any inhibitory properties.

In addition, strain KF525 produced a second structural class of natural products including the new calcarides A–E and five related compounds of the 15G256-type. The compounds are linear and macrocyclic polyesters with the calcarides representing methylated forms of the 15G256-type compounds. The absolute configuration of the metabolites was investigated by measurements of the optical rotation. The calcarides and 15G256-type compounds were found to possess antibacterial activities against *Staphylococcus epidermidis*, *Xanthomonas campestris* and *Propionibacterium acnes* underlying tight structure-activity relationships.

From another fungal strain, *Cladosporium* sp. KF501, members of the family of tropolone fungal metabolites were isolated. Among the compounds was a new

derivative that represents a stereoisomer of the known tropolones of the strain. Single-crystal X-ray diffraction analysis was employed in order to determine the relative configuration of the new derivative. The isolated tropolones showed antibacterial and antifungal properties when profiled against *Xanthomonas campestris* and *Trichophyton rubrum*.

Overall, the results of the study demonstrate the high value of fungi from marine habitats for the discovery of new bioactive natural products.

ABBREVIATIONS

(w/v)	weight per volume
$[\alpha]^{20}_{D}$	specific rotatory power; sodium D-line (589 nm); 20 °C
$[\alpha]^{22}_{D}$	specific rotatory power; sodium D-line (589 nm); 22 °C
°C	degrees Celsius
μ	absorption coefficient (in connection with X-ray crystal data)
1D	one-dimensional
2D	two-dimensional
A	adenylation
Å	ångström
ACP	acyl carrier protein
AT	acyl transferase
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
br	broad
c	concentration
C	condensation
calcd	calculated
CoA	coenzyme A
COSY	correlation spectroscopy
CYC	cyclase
d	doublet
D_{calcd}	calculated density
DEPT	distortionless enhancement by polarization transfer
DH	dehydratase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSMZ	German collection of microorganisms and cell cultures
Em	emission
ER	enoyl reductase
ESI	electrospray ionization
ESIMS	electrospray ionization mass spectrometry
EtOAc	ethyl acetate
EtOH	ethanol

Ex	excitation
FDVA	N^{α}-(2,4-dinitro-5-fluorophenyl)-D-valinamide
g	acceleration of gravity
g	gram
h	hour
HMBC	heteronuclear multiple bond correlation
HPLC	high-performance liquid chromatography
HRESIMS	high-resolution electrospray ionization mass spectrometry
HSQC	heteronuclear single-quantum coherence
Hz	Hertz
IC_{50}	half maximal inhibitory concentration
ITS	internal transcribed spacer region
J	spin-spin coupling constant
K	Kelvin
KR	ketoreductase
KS	ketosynthase
L	liter
m	meter
M	molar concentration
m/*z*	mass-to-charge ratio
MeCN	acetonitrile
MeOH	methanol
MHz	megahertz
MIC	minimal inhibitory concentration
MICs	minimal inhibitory concentrations
min	minute
mp	melting point
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectrometry
MT	methyl transferase
Mult.	multiplicity
MW	molecular weight
NMR	nuclear magnetic resonance
NRPS	nonribosomal peptide synthetase

NRPSs	nonribosomal peptide synthetases
OD_{600}	optical density at 600 nm
PCP	peptidyl carrier protein
PCR	polymerase chain reaction
Phe	phenylalanine
PKS	polyketide synthase
PKSs	polyketide synthases
ppm	parts per million
Pro	proline
PYG	peptone yeast glucose medium
q	quartet
Refl.	reflections
rpm	revolutions per minute
s	singlet
SAM	*S*-adenosylmethionine
sh	shoulder
SI	Supporting Information
T	temperature
t	triplet
TE	thioesterase
t_R	retention time
UV	ultraviolet
V	unit cell volume
WHO	World Health Organization
Z	number of molecules per unit cell
δ	NMR chemical shift [ppm]
ε	molar extinction coefficient
λ_{max}	wavelength of maximum absorption

INTRODUCTION

Fungi – an Attunement

Every year when days get shorter and autumn comes along it is the time that fungi become apparent in the woods. Though edible mushrooms are often the first picture coming to one's mind when thinking of fungi, there is much more fungal existence in our daily lives. Fungi have an impact on humans as yeasts are used for baking and brewage. Fungi are the main pathogens of man's crop plants. It is to their credit that antibiotics were discovered, healing people on the one hand; on the other hand fungi cause diseases such as athlete's foots. A closer look reveals that fungi are present on Earth almost everywhere, e.g., in air as spores, in soil, in ocean and fresh waters, on rocks, on dead organic matter or in associations with plants, animals and microbes including other fungi (Hawksworth and Mueller 2005, Fröhlich-Nowoisky et al. 2009). This diverse distribution is reflected in an estimated number of 1.5 million fungi present on Earth (Hawksworth 2001).

With respect to their biological characteristics, fungi are cell wall possessing, generally non-motil, heterotroph organisms that represent an entire kingdom within the domain of the Eukaryotes. They comprise unicellular forms such as yeasts. Yet the majority of fungi are filamentous organisms possessing a mycelium, which is build up by filaments called hyphae. In addition, some species have the ability to switch between uni- and multicellular forms, a phenomenon referred to as dimorphism. Concerning reproduction, fungi show a haploid, asexual life cycle and a sexual life cycle in which a haploid phase alternates with a diploid phase. Characteristically, both types of reproduction can be observed for most species with the respective forms called teleomorphs (sexual reproductive state) and anamorphs (asexual reproductive state). In the context of metabolic activities, fungi are able to convert a broad variety of complex biological polymers. One outcome of their metabolic processes is the numerously produced, digesting exoenzymes facilitating degradation, e.g., of wood, giving fungi a key function as decomposers of organic material. Beside their primary metabolism, fungi have evolved an extraordinary rich secondary metabolism, a feature they share with plants and bacteria mainly (Bennett and Bentley 1989). The resulting secondary metabolites, often referred to as natural products, were the main study subject of the present PhD thesis.

Fungal Natural Products

Systematic studies of fungal natural products were started in 1923 by Harold Raistrick and colleagues, leading to the isolation of some 200 mold metabolites such as patulin, penicillic acid and geodin (Raistrick 1949). Some years later when Alexander Fleming discovered penicillin G, a real success story of fungal natural products began (Figure 1). Fleming's discovery was an observation by chance as he found an undesired contamination in one of his petri dishes containing a culture of staphylococci in 1928. He noticed that the mold, which he identified as *Penicillium notatum*, inhibited the growth of the bacteria. Fleming characterized penicillin as the mold's active principle which at the same time lacked toxicity on human blood cells and animals (Fleming 1945). It was not until ten years later that Howard W. Florey, Ernest Chain and colleagues were able to prepare sufficient and stable penicillin suitable for clinical trials. With these studies the compound was finally proved a very effective antibiotic (Nicolaou and Montagnon 2008).

Stimulated by the discovery of penicillin G, a new epoch in research for biologically active molecules was ushered in. It turned out that especially molds and actinomycetes bacteria were potent natural product producers (Bérdy 2005). Further antibacterial drugs of fungal origin were developed, e.g., cephalosporin C identified from *Cephalosporium acremonium* (Newton and Abraham 1955) or fusidic acid, which was first isolated from *Fusidium coccineum*, and still provides high activity against resistant *Staphylococcus* strains (Godtfredsen et al. 1962, Aly et al. 2011). The more natural products from fungi were described, the broader the panel of applications became. Griseofulvin from *Penicillium griseofulvum* was one of the first antifungal metabolites used for therapy of dermatophyte infections of skin, hair and nails (Grove et al. 1952, Aly et al. 2011). Cyclosporin, obtained from *Tolypocladium inflatum* in 1971, was found to be a suppressor of T-cells and for the first time provided transplantation surgery with a selective immunosuppressive drug for administration after organ transplantations (Dreyfuss et al. 1976, Grabley and Thiericke 1999). The statins, e.g., lovastatin, which was originally isolated from *Monascus ruber* and later from *Aspergillus terreus*, represent another important example among fungal natural products with use in medicine (Alberts et al. 1980). Statins attracted attention because of their cholesterol-lowering properties leading to the commercialization of lovastatin as an antihypercholesterolemic drug in 1987. Further statins followed the way to the market, especially semisynthetic and totally synthetic analogs derived from the fungal natural

statins of which the compound atorvastatin has been the leading drug of the entire pharmaceutical industry with respect to market share for many years (Demain and Sanchez 2009).

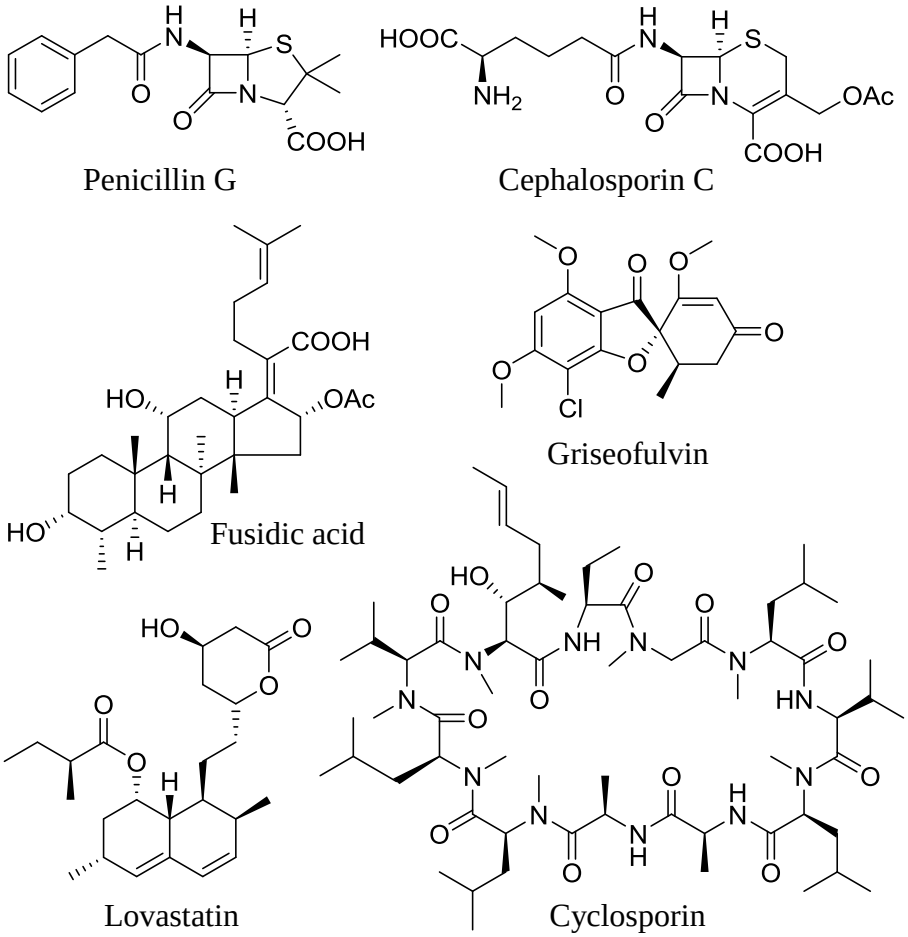

Figure 1. Fungal natural products with therapeutic use in medicine.

Regarding their basic biological features, fungal natural products in the sense of secondary metabolites are defined as chemical structures, which in contrast to required primary metabolites are not essential for the growth of the producing organism (Aharonowitz and Demain 1980). The set of natural products can differ from species to species, meaning that their production is limited to narrow taxonomic groups, sometimes up to the extent that compounds are species-specific (Vining 1990). Typically, the metabolites are produced at the end of the growth phase or in early stationary phase (Laatsch 2000), but exceptions can be observed. From a chemical perspective, natural products are characterized by low molecular weights and at the same time high structural complexity and diversity (Williams et al. 1989, Laatsch 2000). One of Raistrick's colleagues once stated about mold metabolism, "If ever there was a field in which a pentavalent carbon atom could turn up, this is it!" (Raphael

1948), paraphrasing the seemingly unrestricted and partly unimaginable variety found in chemical structures of fungal natural products.

On the basis of their structural diversity, the broad spectrum of biological activities of fungal natural products can be explained. In addition to the above mentioned antibacterial, antifungal, immunosuppressant and antihyperlipidemic examples, a plethora of further fungal compounds showing biological properties has been isolated. The activities range from antiviral, anti-insecticidal, antiprotozoal to antiparasitic (e.g., anthelmintic) effects. Furthermore, activities including cytotoxic, antitumoral, mutagenic, carcinogenic, teratogenic properties and different enzyme inhibitory effects have been assigned to fungal natural products (Keller et al. 2005). Therefore, natural products of fungal origin have a broad field of application not only in human medicine, but also in veterinary medicine and agriculture, e.g., in crop protection.

Though the practical benefit of natural products for humans is evident, their ecological function and relevance in the natural environment often remains elusive. As fungi are sessile organisms, the production of bioactive compounds can be a chemical defense mechanism toward competitors rivaling for the same nutrients and habitats or as an anti-predator adaptation (Kettering et al. 2004, Rohlfs et al. 2007). The fact that natural products are frequently synthesized in a specific stage of developmental processes link compounds and their function to the morphological differentiation of the producing fungi. For instance, pigments known as melanins are associated with fungal spore formation, contributing to the spores' survival by the UV protecting features of melanins (Calvo et al. 2002). Just as well natural products are recognized as mediators of a number of symbiotic and parasitic interactions (Vining 1990). This relationship is exemplified by secondary metabolites of endophytic fungi which are correlated to the increased tolerance toward biotic stress in the fungi-hosting plants (Saikkonen et al. 1998).

For the majority of natural products, the precise ecological function has not been understood though. This is especially true for those compounds that have no demonstrated activity under laboratory conditions. The ecological role of natural products has therefore always been matter of a controversial debate and it has even been questioned whether the compounds are just the result of a "games room" of biochemical evolution (Zähner 1979). However, given that the biosynthesis of natural products involves complex enzyme systems (Laatsch 2000), which are encoded by genes

accounting for many kilobases of DNA (Williams et al. 1989), it is energetically a costly process that is logically only justified, if the compounds provide a benefit to the producing organism.

Biosynthesis of Fungal Natural Products

Despite the chemical diversity of natural products, they are produced by only a few biosynthetic pathways. Studies on the genes encoding fungal natural products revealed that they are usually clustered on a single genetic locus, a feature that was formerly thought to be exclusive of prokaryotic organisms (Keller and Hohn 1997). There are four classes of fungal natural products with regard to their chemical scaffolds which in turn reflect the metabolic route involved in biosynthesis: polyketides, nonribosomal peptides, terpenes and indole alkaloids. The basic principles of polyketide and nonribosomal peptide biosynthesis, being of particular interest in this study, shall be described more in detail here.

Polyketides

Polyketides are a diverse group of natural products, ranging from very simple structures to highly complex ones, including polyphenols, macrolides, polyenes, enediynes and polyethers (Hertweck 2009). The assembly of polyketides is catalyzed by large multi-enzyme complexes called polyketide synthases (PKSs) which use short-chain carboxylic acids, in fungi generally acetyl coenzyme A (CoA) and malonyl CoA, as building blocks. During the enzymatic process, the substrates are successively condensed to build up carbon chains of varying length (Figure 2). A PKS contains a minimal set of three domains that are the catalytic units essential for chain elongation: The acyl transferase (AT) is responsible for the substrate recognition, the acyl carrier protein (ACP) functions as a transporter carrying intermediates through the catalytic cycle and the ketosynthase (KS) catalyzes C–C bond formations between extender units and the growing polyketide chain by decarboxylative Claisen reactions (Keller et al. 2005, Crawford and Townsend 2010). The biochemistry used in PKS systems is similar to that in fatty acid biosynthesis known from the primary metabolism. The two enzymatic systems however differ in that the β-carbon of the extending chain always becomes fully reduced in fatty acid formation, whereas in polyketide biosynthesis β-keto-processing reactions performed by ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains are optional. The level of reduction therefore varies

depending on the polyketide that is built. Additionally, the carbon chain can be modified by cyclase (CYC) and methyl transferase (MT) activities (Schümann and Hertweck 2006, Cox 2007). Despite the simple building blocks used in polyketidic biosynthesis, the programming of their type and number, the number of reduction reactions, extend of methylation, cyclization reactions as well as post-PKS steps give rise to very different end products. The striking diversity of polyketides is hence a result of the combination of these variables allowing innumerable structural possibilities (Keller et al. 2005, Hertweck 2009).

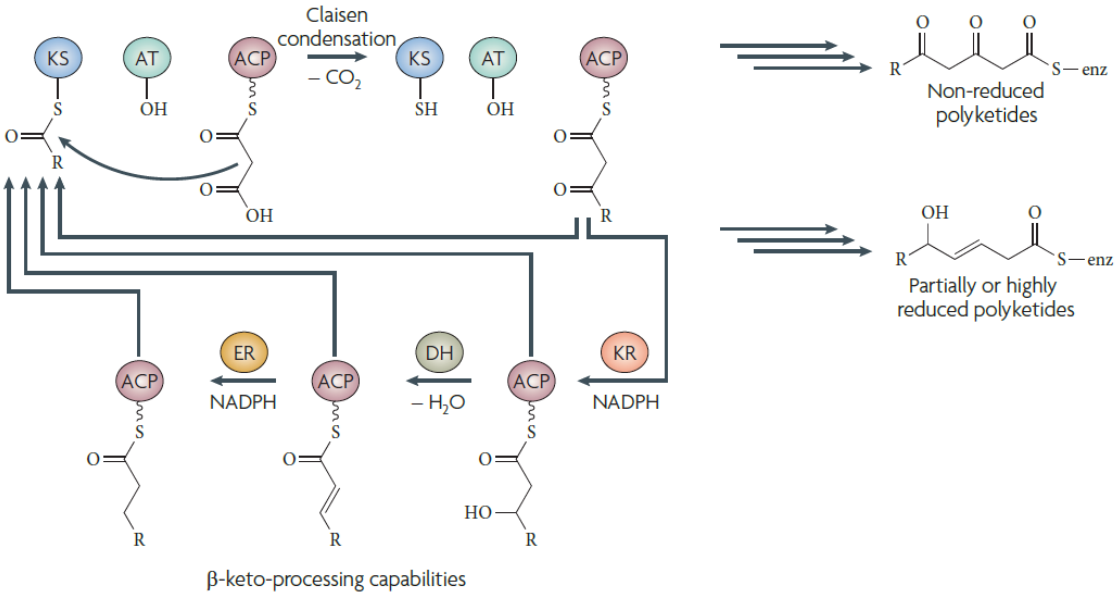

Figure 2. Basic mechanisms of polyketide biosynthesis (modified according to Crawford and Townsend 2010). KS, ketosynthase; AT, acyl transferase; ACP, acyl carrier protein; KR, ketoreductase; DH, dehydratase; ER enoyl reductase; enz; enzyme.

Though the basic mechanisms of polyketide biosynthesis are applicable to all PKSs, they are divided into three major classes according to the enzyme architecture. Type I PKS are large multidomain enzyme systems comprising covalently fused domains that can either show a modular arrangement or work iteratively. In the modular type I system, typically found in bacteria, the catalytic domains form modules of which each is usually responsible for only one elongation cycle. On the contrary, in the iterative type I system, the domains are reused for the catalytic cycles (Hertweck 2009). The majority of fungal PKS are iterative type I systems and are further subdivided into non-reducing, partially reducing or highly reducing PKSs corresponding to the degree of reductive processing that takes place during the assembly (Crawford and Townsend 2010). In type II PKSs, the catalytic activities are present on dissociable proteins that associate to

perform the enzymatic activities. Type II systems are exclusively found in bacteria, e.g., the formation of bacterial aromatic polyketides is catalyzed by type II iterative systems. Finally, type III PKSs are single, iteratively working KS proteins that do not require auxiliary enzymes and are found in plants, bacteria and fungi (Cox 2007, Crawford and Townsend 2010).

Nonribosomal Peptides

Nonribosomal peptides mainly originate from bacteria as well as fungi, comprising well known natural products such as cyclosporins and β-lactam antibiotics like the penicillins (Strieker et al. 2010). As the name suggests, the biosynthetic mechanisms of nonribosomal peptides differ from the ubiquitous protein formation at the ribosomes in primary metabolism. Unlike for ribosomal proteins, where the translation of the genetic code allows the biosynthesis of different proteinogenic end products at a single catalytic site, nonribosomal peptides are the result of product-specific enzymes called nonribosomal peptide synthetases (NRPSs).

In contrast to fungal iterative PKSs, the large multifunctional NRPS enzyme systems have a modular organization. Each module elongates the nascent peptidyl chain by the addition of one specific amino acid. A typical minimal NRPS elongation module comprises an adenylation (A), a peptidyl carrier protein (PCP) and a condensation (C) domain. Analogous to the PKS biosynthesis, the A-domain selects the respective amino acid and activates it in form of an amino acyl adenylate (Finking and Marahiel 2004) (Figure 3). The spectrum of substrates for the A-domain in NRPSs is remarkably broad. Beside the 20 proteinogenic amino acids, also nonproteinogenic amino acids and other carboxylic acids, e.g., D-forms of amino acids or aryl acids, are incorporated into nonribosomal peptides (Fischbach and Walsh 2006). After adenylation, the activated amino acid is transferred to the PCP-domain, which serves as a transporter passing nonribosomal peptide intermediates from one catalytic center to the next. The formation of the C–N amide bonds between PCP-tethered amino acids and peptidyl intermediates of neighboring modules is finally catalyzed by the C-domain of an NRPS module (Finking and Marahiel 2004). At the end of NRPS biosynthesis, a termination module often comprises thioesterase (TE) activity and releases the nonribosomal peptide by either hydrolysis or macrocyclization (Finking and Marahiel 2004). Aside from the enzymatic domains required in NRPS modules, further tailoring reactions like N-, C- and O-methylation, acylation, glycosylation, heterocyclic ring formation and

epimerization can be conducted by optional domains integrated in the NRPSs or stand-alone enzymes (Mootz et al. 2002). According to these biosynthetic mechanisms, the enormous structural diversity found among fungal nonribosomal peptides arises in the wide range of building blocks employed, the length of peptides as well as the various modifying tailoring reactions (Keller et al. 2005).

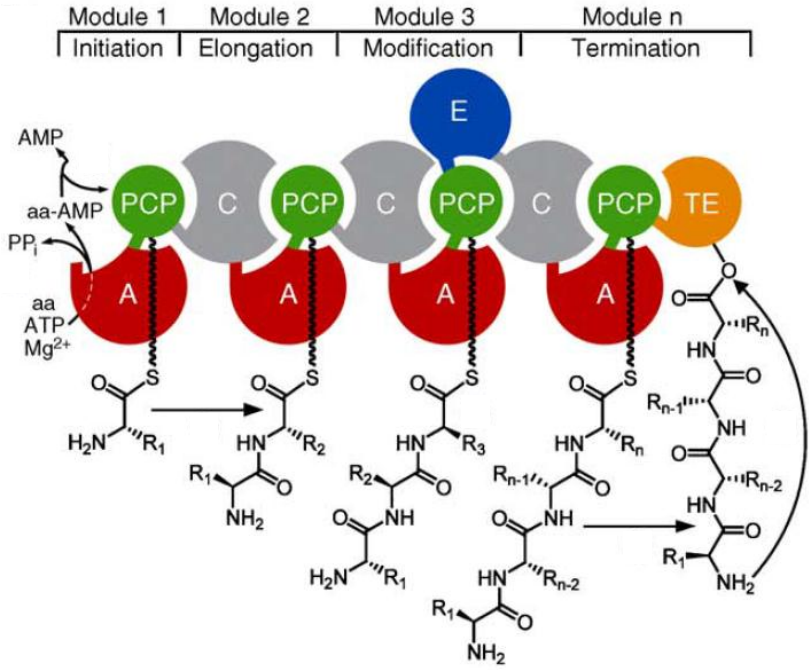

Figure 3. Basic mechanisms of nonribosomal peptide biosynthesis (modified according to Strieker et al. 2010). A, adenylation; PCP, peptidyl carrier protein; C, condensation; TE, thioesterase; E, epimerase; aa, amino acid.

Due to the modular architecture of NRPS systems, the order of elongation modules generally correlates with the order of amino acids incorporated (Hoffmeister and Keller 2007). In these cases, the genes encoding a specific NRPS allow the prediction of the nonribosomal peptide product. However, there is increasing evidence of NRPSs for which this co-linearity is not true, because modules or domains are used more than once during peptide assembly. The NRPS for the fungal trimer enniatin, for instance, comprises a reduced number of modules as the three repeated structural sequences are catalyzed by only one set of modules in an iterative fashion (Glinski et al. 2002, Mootz et al. 2002). A further example for deviation from the classical NRPS systems as described above are a group of compounds called PKS–NRPS hybrids. In the biosynthetic complexes for hybrids, enzymatic activities of PKS and NRPS domains act together, resulting in the production of mixed peptide–polyketide compounds. The first

fungal PKS–NRPS hybrid, fusarin C, was only identified in 2004 (Song et al. 2004) and little is known about their biosynthetic background to date. Therefore, PKS–NRPS hybrids represent an interesting investigation field, probably giving rise to a highly diverse pool of natural products in the future.

Need for New Natural Products

The golden era of antibiotics from 1940 to 1980 (Demain 2013) has brought great benefits to humankind. With the antibiotics discovered in that time, the mortality due to infectious diseases was decreased significantly. Diseases formerly often lethal were now treatable. Microbial and plant natural products helped to almost double humans' life span during the last century (Li and Vederas 2009). On the other side, having very effective drugs to treat infectious and other diseases at hand might have led to an underestimation of the constant need for the search of new drugs. The rate of discovery of new bioactive compounds and new drug approvals has dropped since the late 1990s and slowed down drastically in the 21st century (Demain 2013). One important cause for this decrease was the draw back by the major pharmaceutical companies from their natural product research facilities, in part for reasons of an unbalanced equilibrium between high costs required before a drug becomes approved and low market sales until the products become generics (Demain 2013).

The lack of new therapeutics is dramatic as resistances against the existing drugs are increasing. Ever since antibiotics have been clinically applied, resistances have been observed. This problem is only partly manmade as microbes are faced with naturally occurring antibiotics in their original ecosystems, sometimes being the producers of the compounds themselves. The development of antibiotic resistance mechanisms therefore is an evolutionary consequence to guarantee their survival, no matter whether it happens in the ecological habitat or in a treated patient. However, the extensive administration of antibiotics in the therapy for humans as well as animals has resulted in the accelerated selection of resistant pathogenic strains (Nikaido 2009). The situation becomes even more problematic as genes for drug resistance can be transferred to sensitive strains.

Today not only single-drug resistances make antibacterial treatments difficult – many human pathogens have evolved multidrug resistances (Levy and Marshall 2004). A notorious example is the methicillin-resistant *Staphylococcus aureus* (MRSA). Aside from methicillin-resistance, MRSA strains are usually resistant to aminoglycosides,

macrolides, tetracycline, chloramphenicol and lincosamides, too (Nikaido 2009). In many cases, vancomycin is the antibiotic of choice for methicillin-resistant *S. aureus*, but in the early 2000s clinical specimens with resistance to vancomycin eventually also emerged (Enright 2003, de Lencastre et al. 2007). MRSA strains, as other multidrug-resistant bacteria, are often associated with hospital-acquired infections, so called nosocomial infections, depicting a severe problem in the national health care systems. In 2004, about 70% of the hospital-acquired infections in the United States were caused by pathogens resistant to at least one of the current antibiotics (Infectious Diseases Society of America 2004).

The list of further multidrug resistant pathogens compromising effective antibiotic therapy is long. To name just a few: The causative organisms of tuberculosis, *Mycobacterium tuberculosis* strains, have evolved resistances toward four or more of the front-line treatments and even totally drug-resistant variants have emerged (Velayati et al. 2009, Davies and Davies 2010). As a result, tuberculosis is highly prevalent in the world today, leading to 1.3 million deaths in the year 2012 (Koul et al. 2011, WHO 2013). A more recent serious threat comes from Gram-negative bacterial strains, notably *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, which are sometimes resistant to all, or all but one, antibiotics (Levy and Marshall 2004). Moreover, resistance is also evident in microorganisms other than bacteria responsible for infectious diseases such as viruses, fungi or parasites (Norrby et al. 2005, Pfaller 2012).

Aside from drug resistances, there are newly emerging pathogens for which innovative therapeutic approaches are required. The majority of new human infections have their original source in animals (Jones et al. 2008). The human immunodeficiency virus, for instance, is an emerging infectious agent, which found its way from the original primate host, the chimpanzees, to the human population (Morens and Fauci 2013). Other examples of newly emerging diseases include the severe acute respiratory syndrome and the pandemic H1N1 influenza virus, both of which have caused severe illnesses and deaths in the modern world (Morens and Fauci 2013). After all, infectious diseases remain among the leading causes of death (WHO 2013).

The situation is equally concerning in the field of cancer treatment. Although great advance in cancer medicine has been made, the prognosis for a number of cancers, e.g., pancreatic and lung cancer, is still poor (de Bono and Ashworth 2010). An effective treatment is demanding due to the highly complex biology behind cancer (Luo et al.

2009). Moreover, as for infectious diseases, the appearance of resistance toward therapeutic agents represents an additional complicating component in anticancer therapy (Baguley 2010). It is thus estimated that in 2008 12.7 million people suffered from newly acquired cancer and with 13% of all deaths (7.6 million cases in 2008), cancer is a major cause of death worldwide (Ferlay et al. 2010, WHO 2013).

Given that the chemical variety of current drugs seems not sufficient for today's therapeutic demands in medicine, there is an urgent need for new appropriate drugs. In this regard, natural products play an important role in drug discovery and development as becomes apparent by their statistical significance in the treatment of human diseases (Cragg et al. 1997, Newman et al. 2003, Newman and Cragg 2007, Newman and Cragg 2012). Despite the reduced interest of pharmaceutical companies in natural product research, this field still produced or was involved in approximately 50% of all small molecule approvals in the time from 2000 to 2010. In the area of small-molecule anticancer drugs, natural products or agents directly derived therefrom represent an important proportion (44.4%) of the drugs approved worldwide in the last 30 years. Similarly, drugs originating from natural products accounted for 47.1% of the approvals for small-molecule anti-infectives (bacterial, fungal, parasitic and viral) in the same time period. Among this drug class, an outstanding situation is found for antibacterial drugs where almost 75% of the small-molecule drugs can be traced back to a natural product source. These numbers illustrate that natural products are indispensable for the development of new drugs. Their success in human therapy obviously relies on their unique chemical features as compared to synthetic compounds, possessing more chirality centers and oxygen atoms as well as often bridged polycyclic carbon skeletons and therefore a higher spatial complexity than synthetic agents (Spížek et al. 2010).

Strategies to Discover New Fungal Natural Products

The search for new natural products is challenging, especially because re-isolation of known structures from natural extracts commonly occurs and the supply of compound is often a limiting factor. For this reason, good screening strategies are essential for the successful isolation and characterization of new compounds.

The producing organism certainly plays a crucial role for natural product discovery. With regard to supply of sufficient compound, microbial sources such as bacteria and fungi have moved into the focus of natural product research, since they can be

fermented in large scales in the laboratory, yielding good quantities of compounds without harming the natural environment. While mainly terrestrial fungi and bacteria, e.g., from soil, have been of interest for natural product discovery for a long time, a great fraction of the world's microbial diversity remains to be explored (Harvey 2008, Bérdy 2012). One strategy for the search of new natural products is hence the investigation of microbes from little-explored environments. Marine habitats, for instance, harbor a largely untouched number of microbes and those studied turned out to be prolific producer of new bioactive compounds (Bhatnagar and Kim 2010, Imhoff et al. 2011). The same is true for endophytic microorganisms and in particular endophytes from rainforests represent a huge, unexplored source for natural products (Strobel et al. 2004, Bérdy 2012). In addition, only few taxonomic groups have been examined thoroughly with regard to their bioactive metabolites production. In the case of fungi, these are especially *Penicillium*, *Aspergillus* or *Fusarium* isolates, whereas many other species have been less intensively studied and certainly bear an untapped potential for the production of yet unknown compounds (Bérdy 2005, Dictionary of Natural Products 2012).

For cultivation of microorganisms, a stimulation of natural product biosynthesis by systematically changing the culture conditions has been approved as an effective screening strategy. The parameters can be easily accessible ones including media composition, aeration, pH value, temperature or shape of culture vessels (Bode et al. 2002). The method has not only shown differences in quantities of metabolites produced, but also induced the production of many different natural products from a single strain. For example, the cultivation of *Aspergillus ochraceus* in different culture flasks and the variation of agitation during cultivation (e.g., still cultures, shaken cultures, *Penicillium* flasks, different fermenters) resulted in the formation of 15 additional compounds which were formerly unknown from the strain (Fuchser 1996). Intensive studies on the influence of several culture conditions on microbial metabolism enabled Zeeck and his colleagues to isolate more than 100 natural products belonging to 25 structural classes from just six microorganisms (Bode et al. 2002). Relating to the variation of culture media, an addition of epigenetic modifiers has recently gained increasing attention. Epigenetic modifiers regulate the level of e.g. acetylation and methylation in histones and thereby control the transcriptional accessibility of biosynthetic gene clusters of natural products. The use of culture media treated with epigenetic modifying agents can hence result in a significant change of metabolic

profiles including the production of unknown compounds as has been shown for *Cladosporium cladosporioides* (Williams et al. 2008).

Another special culture set-up are co-cultivation experiments by incubation of fungal strains with other species. Reflecting the role of natural products as signaling molecules in the interaction within a species or among different species, co-cultivations can trigger the activation of specific metabolic pathways. Cueto et al. (2001) applied this approach by employing a mixed fermentation of a *Pestalotia* strain together with a marine bacterium and thereby discovered the new antibacterial compound pestalone. Remarkably, the interplay of the two organisms was essential for pestalone biosynthesis as no production was observed in cultivations of either strain alone.

The aforementioned strategies applied in natural product research are culture-dependent. However, less than 5% of known fungi can be cultured in the laboratory (Bérdy 2012). In this regard, the growing genetic data pool nowadays allows natural product chemists to broaden the screening strategies widely by genome mining including culture-independent techniques. The identification of conserved regions in biosynthetic genes, e.g., PKS and NRPS genes, by polymerase chain reaction (PCR) experiments provides information if an organism has the putative genetic equipment for the formation of natural products. This information is a helpful tool for the decision on whether or not a strain is worth a detailed investigation. In addition, the increasing number of genome sequences from fungi provides insights into the number of biosynthetic gene clusters present in fungal strains. Impressively, the genomic sequence data of several fungal strains proved that the vast majority of biosynthetic gene clusters remains silent under standard fermentation conditions. For instance, *Aspergillus nidulans* harbors biosynthetic genes for 27 polyketides, 14 nonribosomal peptides, one terpene and two indole alkaloids, though considerably less metabolites have been ascribed to the strain even after extensive screening campaigns (Bok et al. 2006). These numbers by far exceeded anticipated expectations for natural product gene clusters in a single strain and promise the discovery of numerous new natural products by molecular tools in the future. Therefore, several possible ways to gain access to the usually silent gene clusters exist.

One feasible approach comprises the activation of silent genes by incorporation and expression of the entire biosynthetic gene cluster in an appropriate heterologous host. Ishiuchi et al. recently cloned five PKS and one NRPS genes from various fungal

species into a yeast expression vector. The subsequent plasmid-based expression in an engineered *Saccharomyces cerevisiae* strain allowed the identification of six polyketides and two nonribosomal peptides, among them the so far undescribed 7-hydroxy-fumiquinazoline F (Ishiuchi et al. 2012). Though further development of the techniques is needed, heterologous expression offers an enormous potential for natural product discovery, in particular for so far uncultured microorganisms from environmental samples.

Alternatively, the biosynthesis of cryptic natural products can be triggered by manipulating regulatory factors of the silent gene clusters. Many biosynthetic gene clusters comprise pleiotropic regulators which can positively or negatively affect gene transcription. By means of overexpression or deletion of the transcriptional factors, the biosynthetic pathways may be switched on (Brakhage and Schroeckh 2011). Lae A, as a good example, is a global regulator of secondary metabolism in *Aspergillus* species. A deletion of *laeA* blocks the expression of gene clusters for sterigmatocystin and penicillin in *A. nidulans* (Bok and Keller 2004). In contrast, overexpression of *laeA* enhances gene expression for a variety of natural products including the antitumor compound terrequinone A, which was previously unknown from this *Aspergillus* species (Bok et al. 2006). In other cases, transcription factors are pathway-specific and sometimes control the expression of all genes in a certain biosynthetic cluster (Scherlach and Hertweck 2009). As a consequence, the overexpression of the pathway-specific regulator genes can lead to an expression of otherwise silent gene clusters. As proof of principle, Bergmann et al. (2007) activated a PKS–NRPS hybrid gene in *A. nidulans* through overexpression of the respective activator gene (*apdR*) and thereby isolated the new PKS–NRPS hybrids aspyridones A and B. Similarly, the exchange of promoters of biosynthetic genes is a possible strategy for natural product discovery. Chiang et al. (2009) replaced the native promoter of a transcription activator in a PKS gene cluster of *A. nidulans* with an inducible promoter. By use of this technique, the group induced the production of the novel polyketide asperfuranone, proving the approach successful.

As generally true, the different screening strategies for natural product discovery all have their advantages and drawbacks. The access to little-explored habitats can be restricted, cultivation of rare microbes is often labor- and time-intensive, genome mining approaches are cumbersome and have so far mainly been performed with only a

few model organisms such as *Aspergillus* species. Thus, the identification of the entire plethora of fungal natural products presumably requires the whole spectrum of possible approaches, further developments of new techniques as well as combinations of the different strategies.

Scope of the Thesis

In the present PhD thesis 109 fungal isolates from the German Wadden Sea were investigated with regard to their natural product biosynthesis. The main scope of the thesis was to identify new and preferably bioactive natural products. In order to achieve this aim, several culture-dependent and molecular approaches approved for natural product discovery were combined, creating a smart overall screening strategy that allowed a comprehensive analysis of the metabolic potential of the fungi.

In detail, the criteria by which fungal isolates were selected for the search of new natural products comprised

- fungal isolates from a little-explored ecosystem
- less studied genera
- genetic potential according to PKS and NRPS gene sequences
- stimulating effects on biosynthesis by variation of culture conditions.

Fungal Isolates from a Little-Explored Ecosystem

The fungal isolates originated from water samples of the German Wadden Sea. The Wadden Sea not only represents a barely investigated environment concerning fungi, but also is a unique and highly dynamic ecosystem which is characterized by the variability of physical and chemical parameters based on seasonal and tidal changes (Liebezeit et al. 1994, Grunwald et al. 2007). As the adaptation of fungi to such an extraordinary environment requires special metabolic abilities, it was assumed that this might also apply for the fungi's natural product biosynthetic capabilities.

Less Studied Genera

The fungal isolates in the study comprised species mainly belonging to ascomycetes as well as some basidiomycetes and zygomycetes. Almost a third of the isolates (30

strains) were affiliated to the genus *Penicillium*. Though Penicillia are prolific producers of bioactive compounds, it is also one of the best investigated fungal genera regarding natural products and therefore the probability to find already characterized compounds is high. To avoid a re-isolation of known natural products, the focus of the screening was set to other, less examined genera or isolates that did not show high similarities to gene sequences deposited at GenBank, being putative new species.

Genetic Potential According to PKS and NRPS Gene Sequences

The fungi's genetic potential to produce polyketides or nonribosomal peptides was investigated by PCR amplification of the respective biosynthetic gene fragments and subsequent sequencing. For PKS genes, degenerative primer pairs (LC1 and LC2c, LC3 and LC5c) for the amplification of the KS-domain were used (Bingle et al. 1999). The primers for NRPS genes amplified fragments of the C-domain (Klotz 2010). On the basis of the PCR and sequencing data, only PKS- and NRPS-positive strains were selected for further investigations of chemical profiles.

Stimulating Effects on Biosynthesis by Variation of Culture Conditions

All 109 isolates were screened for their natural product formation in one standard culture condition (modified Wickerham medium, still cultures for filamentous fungi and shaken cultures for yeasts). In order to induce the activation of gene clusters that were silent under the standard fermentation condition, a selection (29 strains) of less studied genera and PKS-/NRPS-positive isolates was cultured in five different conditions, more precisely, four different media (potato-carrot medium, modified malt extract medium, Czapek medium, casamino acids glucose medium) and shaking conditions in modified Wickerham medium (except for the yeasts of the selection).

The general methodologies employed for the analysis and dereplication of natural products in the extracts from fungal cultures included HPLC, MS and bioactivity screenings. Putative new bioactive compounds were purified by chromatographic means and elucidated in structure on the basis of NMR spectra. The identification of new natural products raised further questions concerning their stereochemistry, bioactivities and biosynthesis which were subsequently investigated.

CHAPTER I

Calcaripeptides A–C, cyclodepsipeptides from a *Calcarisporium* strain

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Abstract

The isolation and structure elucidation of the novel calcaripeptides A (**1**), B (**2**), and C (**3**) and studies on their biosynthetic origin are described. The calcaripeptides were identified from *Calcarisporium* sp. strain KF525, which was isolated from the German Wadden Sea. Compounds **1**−**3** are macrocyclic structures composed of a proline and a phenylalanine residue as well as a nonpeptidic substructure. Structure elucidation was achieved by applying one- and two-dimensional NMR spectroscopy supported by high-resolution mass spectrometry. X-ray crystallography was performed to determine the relative configuration of **1**. The absolute configuration of **1** was assigned by HPLC of the amino acids after hydrolysis of the molecule and derivatization with chiral agents. Studies on the biosynthesis by feeding ^{13}C-labeled substrates revealed that the nonpeptidic part of **1** originates from acetate and L-methionine. The involvement of a hybrid between a polyketide synthase and a nonribosomal peptide synthetase in the biosynthesis of the calcaripeptides is discussed.

Introduction

With the aim to discover new natural products, fungal strains from the German Wadden Sea were analyzed regarding their metabolite profiles. In Wadden Sea habitats often both true marine organisms and organisms from terrestrial and freshwater habitats frequently occur together. The *Calcarisporium* sp. strain KF525 attracted attention because it produced a set of metabolites that could not be identified by extensive searches of the literature and databases. Fungi of the genus *Calcarisporium* show a widespread occurrence on wood (Sutton 1973, Cooper 2005) and leaf litter (Rambelli et al. 2004, Somrithipol and Jones 2006), in plants as endophytic fungi (Gong and Guo 2010, Ji et al. 2004), or in coal spoil tips (Evans 1971). Commonly, *Calcarisporium* spp. are found as mycoparasites or symbionts of higher basidiomycetes and ascomycetes (Sutton 1973, Barnett 1958, Barnett and Lilly 1958, Watson 1955, Carrión and Rico-Gray 2002, Rombach and Roberts 1987). For some mycoparasitic strains of *Calcarisporium* sp., the ability to reduce feed spoiling molds or fungal plant pathogens, like those responsible for mildews, was observed. Hence, the use of *Calcarisporium* culture filtrates as feed-protecting preservatives and as biocontrol agents in crop protection has been discussed (Ji et al. 2004, Carrión and Rico-Gray 2002, Hijwegen 1989, Hijwegen and Verhaar 1993). It is well known that predatory interactions of microbes are often mediated by enzymes or small-molecule compounds and toxins.

Even though this has also been assumed to be the case for the antifungal interplay of *Calcarisporium* species (Hijwegen 1989), few natural products have been described for the genus (Dictionary of Natural Products 2012). Among them are antifungal compounds such as 15-azahomosterols (Chrisp et al. 1990), aurovertins inhibiting mitochondrial ATP synthesis and ATPases (Baldwin et al. 1964, Mulheirn et al. 1974, Osselton et al. 1974), and calcarisporins B1−B4, with calcarisporin B1 showing cytotoxic activity (Yu et al. 2002a, Yu et al. 2002b).

The marine-derived isolate KF525 of *Calcarisporium* sp. was shown to produce a metabolite spectrum different from those of other known *Calcarisporium* strains. The isolation of these metabolites from the fungal mycelium yielded the novel, structurally related calcaripeptides A (**1**), B (**2**), and C (**3**) described here (Figure I.1). Analysis of the NMR spectroscopic data showed the compounds to be macrocyclic structures consisting of two amino acids (proline and phenylalanine) and a nonpeptidic substructure. The nonpeptidic chain varies in structure between the three compounds. The absolute configuration of **1** was determined by X-ray crystallography in combination with the configuration data of the amino acids obtained by HPLC analysis after hydrolysis of the molecule and chemical derivatization. The biosynthesis of **1** was investigated by feeding ^{13}C-labeled precursors.

Results and Discussion

Strain KF525 was identified as a *Calcarisporium* sp. based on morphological characteristics, in particular microscopic examination of the conidia and conidiophores, and on sequence analysis of the internal transcribed spacer region. As the variation of culture conditions can often be reflected in altered metabolite patterns, the influence of four different culture media as well as static and shaking cultivation conditions on the metabolite production of KF525 was tested. As a response to these varied culture conditions, the strain produced diverse metabolite profiles. While known compounds of the genus *Calcarisporium* were not found, new metabolite spectra were identified. An extract obtained from the mycelia of KF525 grown in modified casamino acids glucose medium (Stevens 1974) under shaking conditions was fractionated by preparative HPLC, yielding compounds **1**−**3**.

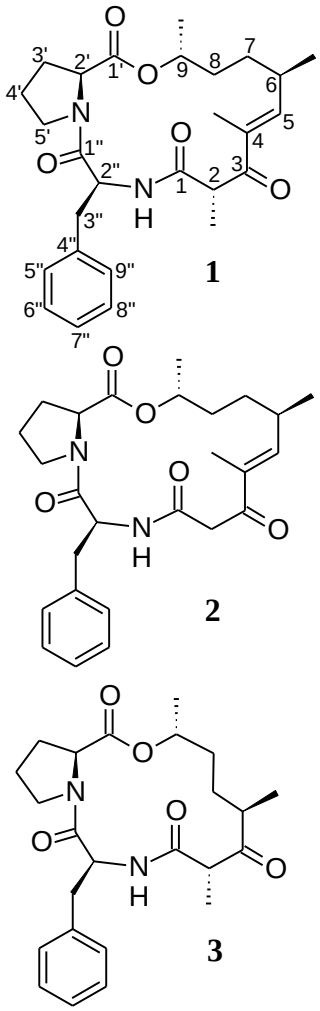


Figure I.1. Structures of calcaripeptides A–C (**1**–**3**), isolated from *Calcarisporium* sp. KF525.

High-resolution ESIMS measurements along with the spectroscopic data gave a molecular formula of $C_{27}H_{36}N_2O_5$ for **1**, requiring 11 degrees of unsaturation. The structure of **1** was established on the basis of one- and two-dimensional NMR spectra (^{1}H, ^{13}C (^{1}H decoupled and DEPT), COSY, HSQC, and HMBC; see Table I.1). The ^{13}C NMR spectrum showed particularly intense signals at δ_C 129.8 and 130.7 accounting for two magnetically equivalent carbons each (C-6″ + C-8″ and C- 5″ + C-9″). These aromatic carbons together with the further aromatic methine signal of C-7″ (δ_C 128.3) and the quaternary carbon C-4″ (δ_C 137.4) gave evidence of a monosubstituted benzene. Four carbonyl functions, C-3 (δ_C 198.6), C-1 (δ_C 174.2), C-1′ (δ_C 171.2), and C-1″ (δ_C 171.8), were observed, of which the latter three had chemical shifts characteristic of amide and ester carbonyl groups. Additionally, the ^{13}C NMR spectrum revealed the presence of two olefinic carbons, C-4 (δ_C 136.6) and C-5 (δ_C 149.6), four methyl groups, six methylene carbons, and five methine carbons.

Table I.1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectroscopic data of calcaripeptide A (**1**) in methanol-d_4.

Position	δ_C, Type	δ_H, Mult. (*J* in Hz)	COSY	HMBC	NOESY[a]
1	174.2, C				
2	49.7, CH	4.23, q (6.9)	2-CH_3	1, 3, 4, 5, 2-CH_3, 2"	5, 2-CH_3
3	198.6, C				
4	136.6, C				
5	149.6, CH	6.42, br d (10.4)	6, 4-CH_3	3, 4, 6, 7, 4-CH_3, 6-CH_3	2, 6, 7a/8a, 7b, 4-CH_3, 6-CH_3, 2"
6	35.2, CH	2.54, m	5, 7b, 6-CH_3	4, 5, 7, 6-CH_3	5, 7a/8a, 8b, 4-CH_3, 6-CH_3
7a	35.7, CH_2	1.40, m[b]		5, 6, 8, 6-CH_3	5, 6, 7b, 9, 6-CH_3, 2"
7b		1.06, m	6, 8a, 8b	5, 8, 9	5, 7a/8a, 9, 2"
8a	35.9, CH_2	1.40, m[b]	7b, 8b, 9	6, 7, 9	5, 6, 7b, 9, 6-CH_3, 2"
8b		1.32, m	7b, 8a, 9	5, 7, 9, 9-CH_3	6, 9
9	75.0, CH	4.65, m	8a, 8b, 9-CH_3	7, 9-CH_3, 1'	7a/8a, 7b, 8b, 9-CH_3
2-CH_3	14.3, CH_3	1.28, d (6.9)	2	1, 2, 3	2, 5" + 9", 6" + 8"
4-CH_3	12.2, CH_3	1.73, d (1.2)	5	3, 4, 5, 6, 7, 6-CH_3	5, 6
6-CH_3	20.7, CH_3	1.01, d (6.6)	6	5, 6, 7, 8	5, 6, 7a/8a
9-CH_3	21.0, CH_3	1.20, d (6.2)	9	8, 9	9, 2"
1'	171.2, C				
2'	60.5, CH	3.92, br d (7.0)	3b'	1', 3', 4', 5'	3a', 3b', 5a', 2", 3a", 3b", 5" + 9"
3a'	28.8, CH_2	2.06, br dd (12.3, 7.0)	3b', 4'	1', 2', 4', 5'	2', 3b', 4'
3b'		1.13, m	2', 3a', 4'	1', 2', 4', 5'	2', 3a', 4', 5a', 5" + 9", 6" + 8", 7"
4'	22.7, CH_2	1.79, m	3a', 3b', 5a', 5b'	2', 3', 5'	3a', 3b', 5a', 5b'
5a'	46.8, CH_2	3.43, dt (11.8, 8.8)	4', 5b'	2', 3', 4', 1"	2', 3b', 4', 5" + 9"
5b'		3.36, ddd (12.2, 9.2, 3.2)	4', 5a'	2', 3', 4', 1"	4'
1"	171.8, C				
2"	53.8, CH	5.27, dd (9.1, 5.6)	3a", 3b"	1, 1", 3", 4"	5, 7a/8a, 7b, 9-CH_3, 2', 3a", 3b", 5" + 9"
3a"	41.4, CH_2	3.04, dd (12.9, 5.6)	2", 3b"	1", 2", 4", 5" + 9"	2', 2", 3b", 5" + 9"
3b"		2.86, dd (12.9, 9.1)	2", 3a"	1", 2", 4", 5" + 9"	2', 2", 3a", 5" + 9"
4"	137.4, C				
5" + 9"	130.7, CH	7.22, br d (8.4)	6" + 8"	2", 3", 7", 5" + 9"	2-CH_3, 2', 3b', 5a', 2", 3a", 3b"
6" + 8"	129.8, CH	7.31, br dd (8.4, 7.3)	5" + 9", 7"	4", 6" + 8"	2-CH_3, 3b'
7"	128.3, CH	7.25, br t (7.3)	6" + 8"	5" + 9"	3b'

[a]The NOESY NMR spectrum was recorded at 500 MHz. [b]Proton signals of 7a and 8a overlap.

The ^{1}H NMR spectrum showed five aromatic protons (Table I.1), corroborating the monosubstituted benzene already deduced from the ^{13}C NMR spectrum. HMBC

correlations of H_2-3″ (δ_H 2.86 and 3.04) to the aromatic carbons (C-4″, C- 5″ + C-9″) as well as to C-2″ and C-1″ established a phenylalanine residue as a partial structure of **1**. The COSY spectrum displayed couplings between H_2-3′ (δ_H 1.13 and 2.06), H_2-4′ (δ_H 1.79), and H_2-5′ (δ_H 3.36 and 3.43) indicating three consecutive methylene groups, characteristic of a proline residue. In addition, correlations of H-2′ (δ_H 3.92) to H-3b′ in the COSY spectrum and to C-1′ in the HMBC spectrum evidenced that C-2′ was the proline α-carbon. The amide linkage between the phenylalanine and proline residues was proven by an HMBC correlation of H_2-5′ to C-1″ and is consistent with the chemical shift of C-1″ (δ_C 171.8). A coupling of H-9 to C-1′ indicated a connection between CH-9 and C-1′, which was further characterized as an ester bond by the respective chemical shifts (δ_C 75.0, δ_H 4.65, and δ_C 171.2). The COSY spectrum revealed that H-9 was part of a proton spin system that reached from 9-CH_3 (δ_H 1.20) to H-5 (δ_H 6.42) including the methyl group 6-CH_3. According to the chemical shifts, CH-5 (δ_C 149.6, δ_H 6.42) was olefinic. The methyl-substituted, olefinic carbon C-4 had to be located adjacent to CH-5 due to long-range H,C-couplings of H-5 to C-4 and 4-CH_3 and of 4-CH_3 (δ_H 1.73) to C-4 and C-5. The *E*-configuration of the double bond was determined on the basis of the NOESY spectrum, in which 4-CH_3 showed a more intense cross-peak with H-6 than with H-5. In addition, the configuration was supported by a NOESY cross-peak between 6-CH_3 and H-5. HMBC correlations of H-2 (δ_H 4.23) to 2-CH_3, C-1, and C-3 as well as correlations of H-5 and 4-CH_3 to C-3 completed the consecutive chain from C-1 to C-9. Finally, correlations of H-2 to C-2″ and H-2″ to C-1 linked C-1 to the phenylalanine residue via an amide bond.

The aromatic ring, the proline ring, the macrocycle, four carbonyl groups, and the double bond $\Delta^{4,5}$ accounted for 11 degrees of unsaturation, as was required by the molecular formula of **1**. Thus, a cyclodepsipeptide structure was established for **1**.

A single-crystal X-ray diffraction analysis of a sample recrystallized from MeOH confirmed the structure of **1** and established its relative configuration. It was observed that the asymmetric unit of **1** consists of five different crystallographically distinct molecules with identical relative configuration. Small differences are found only in the conformation of each molecule. Figure I.2 shows one of these molecules as a representative. The absolute configuration of **1** was then deduced from the configurations of the phenylalanine and proline residues. Their configurations were assigned by HPLC analyses of their D-FDVA (N^{α}-(2,4-dinitro-5-fluorophenyl)-D-

valinamide) derivatives (advanced Marfey's method) after hydrolysis of the molecule. As comparative standards, commercially available L-amino acids were derivatized with D-FDVA and L-FDVA. By comparison of the sample and standards, both the phenylalanine and the proline residue were proven to be L-configured. Combining this information with that of the X-ray analysis, the absolute configurations at all stereogenic centers of **1** were determined as follows: 2*S*, 6*R*, 9*R*, 2′*S*, and 2″*S*; the conformation of the proline amide bond was *cis*.

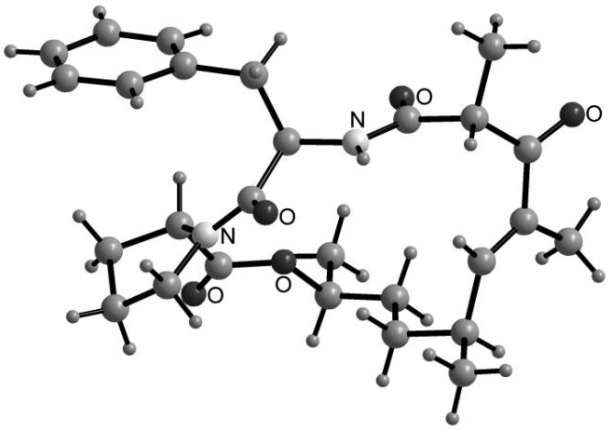


Figure I.2. Molecular structure of one of the five crystallographically distinct molecules in the crystal structure of **1**.

The NMR spectra of **2** were very similar to those of **1** (Table I.2). Because the spectra of **2** were recorded in the aprotic solvent acetone-d_6 instead of methanol-d_4, an additional signal at δ_H 7.51 appeared in the ^{1}H NMR spectrum of **2** corresponding to the amide proton NH-2″. Couplings between NH-2″ and CH-2″ in the COSY spectrum confirmed their vicinity, as was already deduced from the spectroscopic data of **1**. Furthermore, the NMR spectra of **2** lacked the signal of the methyl group at C-2, which was fully consistent with a mass difference of 14 between structures **1** and **2**. In accordance with the loss of the methyl group, CH_2-2 was a methylene group. The signals of H-2a and H-2b (δ_H 4.32 and 3.15) showed low intensities, and the carbon signal of C-2 (δ_C 48.2) was weak. Therefore, its chemical shift had to be deduced from the HSQC spectrum. The low signal intensities can be ascribed to the acidic nature of CH_2-2 being in the α-position of a β-ketoamide function. The absolute configuration of **2** was postulated in analogy with **1**, yet it was not empirically confirmed.

The NMR spectra of **3** proved that its structure was almost identical to **1**, except for the lack of the methyl-substituted double bond (Table I.2). This was in agreement with a

mass decrease of 40 compared to **1**, accounting for C_3H_4. The structure of **3** was confirmed by the analysis of the two-dimensional NMR spectra. The absolute configuration of **3** was also postulated in analogy with **1**.

Table I.2. ^{1}H and ^{13}C NMR spectroscopic data of calcaripeptide B (**2**) (500 MHz and 125 MHz) and calcaripeptide C (**3**) (600 MHz and 150 MHz) in acetone-d_6.

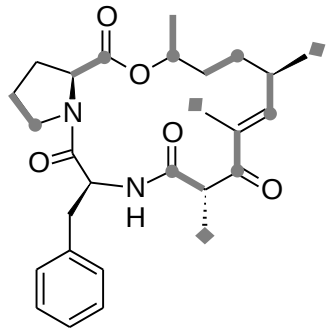
	Calcaripeptide B (2)		**Calcaripeptide C (3)**	
Position	**δ_C, Type**	**δ_H, Mult. (*J* in Hz)**	**δ_C, Type**	**δ_H, Mult. (*J* in Hz)**
1	167.5, C		171.0, C	
2a	48.2, CH_2[a]	4.32, br s	52.3, CH	3.66, q (6.9)
2b		3.15, br s		
3	196.3, C		209.3, C	
4	136.0, C		44.1, CH	2.85, m
5a	149.4, CH	6.40, br d (10.1)	31.8, CH_2	1.53, m[b]
5b				1.06, m
6a	34.6, CH	2.53, m	35.3, CH_2	1.67, m
6b				1.53, m[b]
7a	35.4, CH_2	1.44, m[b]	74.0, CH	4.84, m
7b		1.15, m		
8a	35.6, CH_2	1.48, m[b]		
8b		1.32, m		
9	74.1, CH	4.63, m		
2-CH_3			14.5, CH_3	1.22, d (6.9)
4-CH_3	11.6, CH_3	1.70, d (1.3)	15.6, CH_3	1.02, d (6.7)
6-CH_3	20.6, CH_3	0.98, d (6.6)		
7-CH_3			21.0, CH_3	1.19, d (6.3)
9-CH_3	20.9, CH_3	1.18, d (6.2)		
1'	171.2, C		172.2, C	
2'	59.7, CH	3.85, br d (7.5)	59.8, CH	3.90, m
3a'	29.0, CH_2	2.01[c]	30.3, CH_2	1.87, m
3b'		1.29, m		1.46, m
4a'	22.5, CH_2	1.77, m	22.4, CH_2	1.88, m
4b'				1.71, m
5a'	46.2, CH_2	3.39, m	46.3, CH_2	3.38, m
5b'		3.34, m		
1"	170.6, C		169.6, C	
2"	53.4, CH	5.18, m	54.2, CH	4.97, m
3a"	41.2, CH_2	3.08, dd (12.8, 5.0)	40.6, CH_2	3.06, ddt (12.5, 4.7, 2.5)
3b"		2.80, dd (12.8, 9.0)		2.93, dddd (12.5, 10.0, 3.2, 2.1)
4"	137.7, C		138.2, C	
5" + 9"	130.3, CH	7.20, br d (8.4)	130.2, CH	7.25, br d (7.5)
6" + 8"	129.3, CH	7.31, br dd (8.4, 7.4)	129.2, CH	7.30, br t (7.5)
7"	127.7, CH	7.24, br t (7.4)	127.5, CH	7.23, br t (7.5)
NH-2"		7.51, br d (6.7)		7.61, br d (7.5)

[a]Signal deduced from the HSQC spectrum. [b]Proton signals of overlap. [c]Signal partially obscured.

Structurally, the calcaripeptides are related to acremolides A and B (Ratnayake et al. 2008). The compounds share the feature of being cyclodepsipeptides containing an L-proline-L-phenylalanine moiety that together with a nonpeptidic partial structure forms the macrocycle. However, the nonpeptidic part of the acremolides and calcaripeptides differs, and the acremolides possess an additional seven-membered alkyl side chain connected to the ring. The 16- and 14-membered rings of **1**, **2**, and **3** are unusual for natural products.

The calcaripeptides were tested for activities against five bacterial test strains, three fungal test strains, one oomycete, and two cell lines as well as for inhibition of selected enzyme targets (glycogen synthase kinase-3β, acetylcholinesterase, phosphodiesterase 4B2, and protein tyrosine phosphatase 1B). In addition, **1** was tested in further assays including 24 cell lines. Despite the broad panel of 43 assays, neither antibacterial, antifungal, and cytotoxic properties nor inhibition of the enzyme targets could be detected for the calcaripeptides (data not shown; for information on test strains, cell lines and enzymes see the Supporting Information (SI)).

Judging from the structure, a polyketidic biosynthetic origin of the nonpeptide substructure of the calcaripeptides was assumed. Polyketides are a structurally diverse class of natural products synthesized by polyketide synthases (PKS). The substrates of PKS enzymes are CoA thioesters of small carboxylic acids, such as acetate and malonate. In order to prove the suggested biosynthetic pathway of **1**, feeding experiments using ^{13}C-labeled precursors were carried out. The feeding experiment with 1-^{13}C-acetate led to an enrichment of the ^{13}C signals in the positions 1, 3, 5, 7, and 9 (Figure I.3). Therefore, the chain that connects the carboxy group of the L-proline residue with the amino group of the L-phenylalanine residue is built up by five acetate units with the biosynthesis beginning at 9-CH_3 and progressing toward C-1. In addition, a slight enhancement of the ^{13}C NMR signal intensities for C-1′ and C-5′ was observed, revealing two acetate building blocks for the L-proline moiety. The incorporation of ^{13}C-labeled acetate into the L-proline substructure shows that a portion of the amino acid was synthesized *de novo*, and the labeling pattern is consistent with its formation from α-ketoglutarate. The methyl groups of **1**, 2-CH_3, 4-CH_3, and 6-CH_3, originate from *S*-adenosylmethionine (SAM), as was confirmed by the enhancement of their ^{13}C NMR signals after feeding L-methionine-methyl-^{13}C (Figure I.3).



feeding with	enhancement of (enrichment)
1-^{13}C-acetate	C-1 (3.6%), C-3 (3.3%), C-5 (3.8%), C-7 (3.0%), C-9 (3.2%), C-1' (1.4%), C-5' (1.5%)
Me-^{13}C-methionine	2-CH_3 (63.7%), 4-CH_3 (62.3%), 6-CH_3 (60.5%)

Figure I.3. Biosynthetic origin of calcaripeptide A (**1**) as determined by ^{13}C-labeling. For detailed information on the calculation of the ^{13}C enrichment see SI.

According to the feeding studies, a polyketidic origin of the nonpeptidic part of the calcaripeptides was confirmed. The overall structure and the biosynthetic origin of the building blocks support the involvement of a hybrid between a polyketide synthase and nonribosomal peptide synthetase for the peptidic backbone (PKS−NRPS hybrid) in the formation of the calcaripeptides. An increasing number of fungal compounds have recently been shown to be synthesized by this type of enzyme, e.g., fusarin C, aspyridone, or pseurotin (Collemare et al. 2008). The genome of *Calcarisporium* sp. strain KF525 is currently under investigation with the aim of identifying genes encoding the respective PKS−NRPS hybrids responsible for the biosynthesis of the calcaripeptides. Reference sequences of the known hybrid PKS−NRPS genes from other fungi are available for comparison.

Promiscuity with respect to the amino acid substrates has been shown for fungal NRPS *in vitro* and *in vivo* (Qiao et al. 2011, Krause et al. 2001, Xu et al. 2007). As promiscuity might thus be a possibility for the postulated NRPS component of the biosynthesis of **1**−**3**, it was tested whether a supply of structurally related, alternative amino acids as substrates for the NRPS would lead to derivatives of **1**. Supplementation of L-tryptophan, L-tyrosine, or L-histidine to the culture broth of KF525 did not result in

their incorporation into **1** in place of L-proline or L-phenylalanine. Therefore, the enzymes involved in the biosynthesis of the calcaripeptides seem to be specific for their amino acid substrates. Additional supply of L-proline and L-phenylalanine as the naturally incorporated amino acids led to an increased production of **1**, displaying potential for an optimization in the fermentation process.

Experimental Section

General Experimental Procedures

Melting points were determined on an Electrothermal melting point apparatus. Optical rotation measurements were performed on a Perkin-Elmer model 241 polarimeter. UV spectra were obtained on a Perkin-Elmer Lambda 2 spectrophotometer. NMR spectra were recorded on Bruker DRX 500 (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively) and Bruker AV 600 spectrometers (600 and 150 MHz for ^{1}H and ^{13}C NMR, respectively), using the residual solvent signals as internal references (δ_H 3.31 and δ_C 49.0 for methanol-d_4; δ_H 2.05 and δ_C 29.8 for acetone-d_6). Measurements of high-resolution mass spectra were conducted on a benchtop time-of-flight spectrometer (micrOTOF II, Bruker) with positive ESI. Analytical reversed-phase HPLC-UV/MS experiments were carried out on a VWR-Hitachi LaChrom Elite system consisting of an L-2130 pump, an L-2450 diode array detector, an L-2200 autosampler, an L-2300 column oven, and a Phenomenex Onyx Monolithic column (C18, 100 × 3.00 mm) applying an H_2O (A)/ MeCN (B) gradient with 0.1% HCOOH added to both solvents (gradient: 0 min 5% B, 4 min 60% B, 6 min 100% B; flow 2 mL min^{-1}). For mass detections the HPLC system was coupled to an ESI-ion trap detector (Esquire4000, Bruker Daltonics). Preparative HPLC was performed on a VWR LaPrep system equipped with a P110 pump, a P311 UV detector, a Labocol Vario-2000 fraction collector (LABOMATIC), a Smartline 3900 autosampler (Knauer), and a Phenomenex Gemini-NX column (10 µ C18, 100A, Axia, 100 × 50.00 mm). Further compound purifications were conducted on a Merck-Hitachi LaChrom Elite HPLC system consisting of an L-7150 pump, an L-2450 diode array detector, an L-2200 autosampler, and a Phenomenex Gemini-NX column (5 µ C18, 110A, Axia, 100 × 21.20 mm). The eluents for all preparative HPLC separations were H_2O (A) and MeCN (B) with 0.1% HCOOH added to both solvents.

Isolation and Identification of the Fungal Strain

The strain KF525 was isolated from a water sample collected in the German Wadden Sea. The DNA extraction, the amplification of the internal transcribed spacer region (ITS), and the sequencing were performed using standard protocols (Wiese et al. 2011) modified in that the centrifugation was carried out at 8000*g* for DNA extraction, DreamTaq Green PCR Master Mix (2×) (Fermentas) was employed for amplification, 35 cycles instead of 30 were conducted for amplification, and the primer ITS4 was used for sequencing. The DNA sequence was deposited in GenBank under the accession number KC800713. A sequence analysis in GenBank using the Basic Local Alignment Search Tool (BLAST) gave 91% similarity to *Calcarisporium arbuscula*. A microscopic analysis of the strain showed structures typical of the genus *Calcarisporium*. Taken together, the sequence and morphological data allowed the identification of strain KF525 as a *Calcarisporium* sp., as was additionally confirmed by the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands).

Fermentation

Cultivation experiments were performed in 16 2-L Erlenmeyer flasks, each containing 750 mL of modified casamino acids glucose medium (2.5 g of casein hydrolysate, 40 g of glucose × H_2O, 0.1 g of $MgSO_4 \times 7H_2O$, 1.8 g of KH_2PO_4 per liter of distilled H_2O, pH 6.8) (Stevens 1974). Cultures were inoculated with a circular agar slant (1.8 cm in diameter) of a preculture grown on solid modified Wickerham medium (3 g of malt extract, 3 g of yeast extract, 5 g of peptone from soymeal, 10 g of glucose × H_2O, 30 g of NaCl, 15 g of agar per liter of distilled H_2O, pH 6.25) (Wickerham 1951). The preculture was incubated at room temperature in the dark for 11 days. The main cultures were incubated at 22 °C under shaking conditions (120 rpm) in the dark for 24 days.

Purification of Calcaripeptides A−C

The culture broth of KF525 was separated into the culture supernatant and the mycelium. The culture filtrate was extracted with EtOAc. The organic solvent was evaporated to dryness *in vacuo* to give 0.92 g of extract. The extract was fractionated by preparative HPLC on a VWR LaPrep system (gradient: 0 min 10% B, 17 min 60% B, 22 min 100% B; flow 100 mL min^{-1}; UV detection at 217 nm), yielding three fractions that contained the calcaripeptides (t_R 15.7, 16.6, and 17.6 min). These fractions were

further purified on a Merck-Hitachi LaChrom Elite system to give 117.9 mg of **1** (isocratic: 42% B; flow 18 mL min^{-1}; UV detection at 220 nm; t_R 10.7 min), 6.2 mg of **2** (isocratic: 29% B; UV detection at 205 nm; t_R 27.5 min), and 8.5 mg of a compound mixture containing **3** (gradient: 0 min 40% B, 13 min 65% B; flow 18 mL min^{-1}; UV detection at 205 nm; t_R 6.2 min). Compound **3** was subjected to a third preparative HPLC purification on the same system (gradient: 0 min 40% B, 13 min 80% B; flow 18 mL min^{-1}; UV detection at 205 nm; t_R 5.8 min), resulting in a yield of 5.7 mg.

Calcaripeptide A (1): white needles or amorphous solid (MeOH); mp 199−201 °C; $[\alpha]^{20}_D$ −133 (*c* 1.4, MeOH); UV (MeOH) λ_{max} (log ε) 201 (4.43), 230 (sh) (4.14) nm; 1D and 2D NMR data, see Table I.1; HRESIMS *m*/*z* 469.2707 $[M + H]^+$ (calcd for $C_{27}H_{37}N_2O_5$, 469.2697).

Calcaripeptide B (2): colorless oil; $[\alpha]^{20}_D$ −113 (*c* 0.31, MeOH); UV (MeOH) λ_{max} (log ε) 201 (4.35) nm, 230 (sh) (4.09); 1D and 2D NMR data, see Table I.2 and SI; HRESIMS *m*/*z* 455.2538 $[M + H]^+$ (calcd for $C_{26}H_{35}N_2O_5$, 455.2541).

Calcaripeptide C (3): colorless oil; $[\alpha]^{20}_D$ −79 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 201 (4.24) nm; 1D and 2D NMR data, see Table I.2 and SI; HRESIMS *m*/*z* 429.2379 $[M + H]^+$ (calcd for $C_{24}H_{33}N_2O_5$, 429.2384).

X-ray Crystal Structure Determination

Data collection was performed using an imaging plate diffraction system (IPDS-2) from STOE & CIE at 293 K using Mo Kα radiation (γ = 0.71073 Å). Formula: $C_{27}H_{36}N_2O_5$, molecular weight: 468.58 g mol^{-1}, monoclinic, space group *C*2; unit cell dimensions: *a* = 44.5917(12) Å, *b* = 9.6204(2) Å, *c* = 37.0616(10) Å, β = 122.453(2)°, *V* = 13416.1(6) $Å^3$, *Z* = 20, D_{calcd} = 20, 1.160 Mg/m^3, μ = 0.080 mm^{-1}. The structure was solved with methods using SHELXS-97, and refinement was performed against F^2 using SHELXH. All non-hydrogen atoms were refined anisotropically. The H atoms were positioned with idealized geometry and refined isotropically with $U_{iso}(H) = 1.2U_{eq}(C,N)$ using a riding model (1.5 for methyl H atoms). A total of 27 668 reflections were measured in the range of 2θ 1.2−24.6°, of which 11 536 are independent (R_{int} = 0.0347), 1531 parameters, GOF = 1.080, R_1 for 8490 reflections with $I > 2\sigma(I)$ = 0.0521, wR_2 for all reflections = 0.1205. Residual electron density = 0.326/0.164 e/$Å^3$. In one of the five independent molecules slightly enlarged anisotropic displacement parameters are observed for some C atoms, indicating disorder that cannot be resolved successfully.

Because no strong anomalous scattering atoms are present, the absolute structure and absolute configuration cannot be determined, and therefore, Friedel equivalents were merged in the refinement.

Crystallographic data for **1** have been deposited with the Cambridge Crystallographic Data Centre (CCDC-943694). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

Preparation and Analysis of D- *and* L*-FDVA Derivatives*

The hydrolysis of **1** was achieved by dissolving 2.7 mg of the compound in 1.5 mL of 6 N HCl and subsequent heating at 110 °C overnight. The reaction mixture was then concentrated to dryness and redissolved in 250 µL of H_2O. For the derivatization, 50 µL of the hydrolysate solution was mixed with 100 µL of a 1% (w/v) solution of D-FDVA (N^{α}-(2,4-dinitro-5-fluorophenyl)-D-valinamide) in acetone. After addition of 40 µL of 1 M $NaHCO_3$ and 70 µL of DMSO, the mixture was incubated at 60 °C for 2 h. The reaction was stopped by addition of 30 µL of 2 M HCl. The amino acid standards (50 mM L-proline and L-phenylalanine) were derivatized with D-FDVA and L-FDVA in the above-described manner. Prior to analytical HPLC-UV/MS analyses the reaction mixtures were diluted 100-fold with MeOH/H_2O (1:1). The retention times (min) of the amino acid standard derivatives were as follows: D-FDVA-L-Pro (3.69), L-FDVA-L-Pro (3.48), D-FDVA-L-Phe (4.50), and L-FDVA-L-Phe (4.07). The HPLC analysis of the hydrolysate D-FDVA derivatives showed peaks at 3.69 and 4.50 min. An additional confirmation of the amino acid configuration was accomplished by spiking the hydrolysate derivatives with the amino acid standard derivatives.

Biosynthetic Studies

For the feeding experiments, *Calcarisporium* sp. strain KF525 was cultivated as described in the Fermentation Section above. Precultures were 7 to 15 days old. Each feeding experiment was performed in one 2-L Erlenmeyer flask, containing 750 mL of medium. After five days of cultivation ^{13}C-labeled compounds were added as sterile filtered, aqueous solutions (500 mg of sodium acetate-1-^{13}C, Isotec or 250 mg of L-methionine-methyl-^{13}C, Cambridge Isotope Laboratories). For the extraction, the supernatant and the mycelium of the cultures were separated after 21 days of

incubation. The culture filtrate was extracted with EtOAc as described above, while the mycelium was extracted with EtOH. The culture filtrate fed with ^{13}C-labeled acetate was purified on a Merck-Hitachi LaChrom Elite system (gradient: 0 min 28% B, 30 min 61% B; flow 18 mL min^{-1}; UV detection at 230 nm; t_R 17.9 min) to give 8.2 mg of **1**.

In the feeding experiment with ^{13}C-labeled methionine, compound **1** was isolated from the extracts of both the culture filtrate and the mycelium. Afterward, purified **1** was combined for NMR spectroscopy studies. The extract of the culture filtrate was separated on a Merck-Hitachi LaChrom Elite system (gradient: 0 min 28% B, 20 min 50% B; flow 18 mL min^{-1}; UV detection at 230 nm; t_R 14.7 min), yielding 1.6 mg of **1**. The purification of compound **1** from the extract of the mycelium was conducted on the same system (gradient: 0 min 35% B, 20 min 50% B, 20.5 min 70% B, flow 18 mL min^{-1}; UV detection at 230 nm; t_R 12.7 min) with a yield of 0.6 mg.

Feeding Alternative Amino Acids

Each amino acid experiment was performed in one 2-L Erlenmeyer flask, containing 750 mL of medium. Culture conditions and medium were the same as described in the Fermentation Section above. After five days of cultivation the respective amino acid was added as a sterile filtered, 50 mL aqueous solution (end concentration in culture medium: 1 g L^{-1}). The added amino acids were L-tryptophan, L-tyrosine, L-histidine, L-proline, L-phenylalanine, and L-proline plus L-phenylalanine. L-Tyrosine did not dissolve completely, and only the dissolved portion was added to the culture. Two cultures without additionally supplied amino acids served as controls in the experiment. The culture filtrate was extracted with EtOAc as described above, while the mycelium was extracted with EtOH. The extracts were analyzed by analytical HPLC-UV/MS.

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CHAPTER II

Calcarides A–E, antibacterial macrocyclic and linear polyesters from a *Calcarisporium* strain

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Abstract

Bioactive compounds were detected in crude extracts of the fungus, *Calcarisporium* sp. KF525, which was isolated from German Wadden Sea water samples. Purification of the metabolites from the extracts yielded the five known polyesters, 15G256α, α-2, β, β-2 and π (**1**–**5**), and five new derivatives thereof, named calcarides A–E (**6**–**10**). The chemical structures of the isolated compounds were elucidated on the basis of one- and two-dimensional NMR spectroscopy supported by UV and HRESIMS data. The compounds exhibited inhibitory activities against *Staphylococcus epidermidis*, *Xanthomonas campestris* and *Propionibacterium acnes*. As the antibacterial activities were highly specific with regard to compound and test strain, a tight structure-activity relationship is assumed.

Introduction

Fungal species of the genus, *Calcarisporium*, have a widespread occurrence and are frequently found as mycoparasites or symbiotes of higher fungi (Sutton 1973, Somrithipol and Jones 2006, Ji et al. 2004, Kramer et al. 1959, Watson 1955). The *Calcarisporium* sp. strain KF525 investigated in this study was isolated from a water sample taken in the German Wadden Sea. It showed a diverse chemical profile, including ten structurally closely related compounds of the 15G256-type known as, e.g., 15G256α (**1**) or α-2 (**2**) (Figure II.1; to be differentiated from the structural class comprising 15G256γ, δ and ε).

The 15G256-type compounds are produced by a number of fungi, such as ascomycetes, like *Hypoxylon*, *Penicillium*, *Talaromyces*, *Acremonium* and *Scedosporium* species, as well as by the basidiomycete, *Albatrellus confluens* (Schlingmann et al. 2002, Breinholt et al. 1993, Ito et al. 1992a, Roy et al. 1996, Kondo et al. 1993, Arai et al. 2002, Zhou et al. 2009). Since some metabolites were isolated independently from the different producer strains, renaming occurred among the 15G256-type compounds. 15G256α (**1**) is synonymous with BK233-A and NG-012, 15G256α-1 is a synonym of BK233-B and probably NG-011 (discussed in Schlingmann et al. 2002), while 15G256β (**3**) was also named orbuticin, BK233-C and BE-26263 (Schlingmann et al. 2002, Breinholt et al. 1993, Roy et al. 1996, Kondo et al. 1993, Ito et al. 1992b). With regard to the chemical structures, the compounds are polyesters that occur either in closed ring structures as macrocycles or as linear molecules (Figure II.1). Schlingmann et al. (2002) proposed a

biosynthesis route in which 6-hydroxymellein and β-hydroxybutyric acid (Figure II.2) are the precursors of the 15G256 components, forming the acyclic polyesters in the first instance. In later biosynthetic steps, the macrocyclic 15G256 compounds arise from the linear polyesters by ring closure reactions (Schlingmann et al. 2002). Various biological activities have been ascribed to the 15G256 agents, including antifungal, estrogenic and cytotoxic properties, as well as they were shown to potentiate nerve growth factor-induced neurite outgrowth (Schlingmann et al. 2002, Breinholt et al. 1993, Ito et al. 1992a, Roy et al. 1996, Kondo et al. 1993). For example, 15G256α (**1**), α-1 and β (**3**) attracted attention in the field of crop protection, as they displayed antifungal properties against important plant pathogenic fungi, e.g., *Botrytis cinerea* or *Monilinia fructigena* (Breinholt et al. 1993).

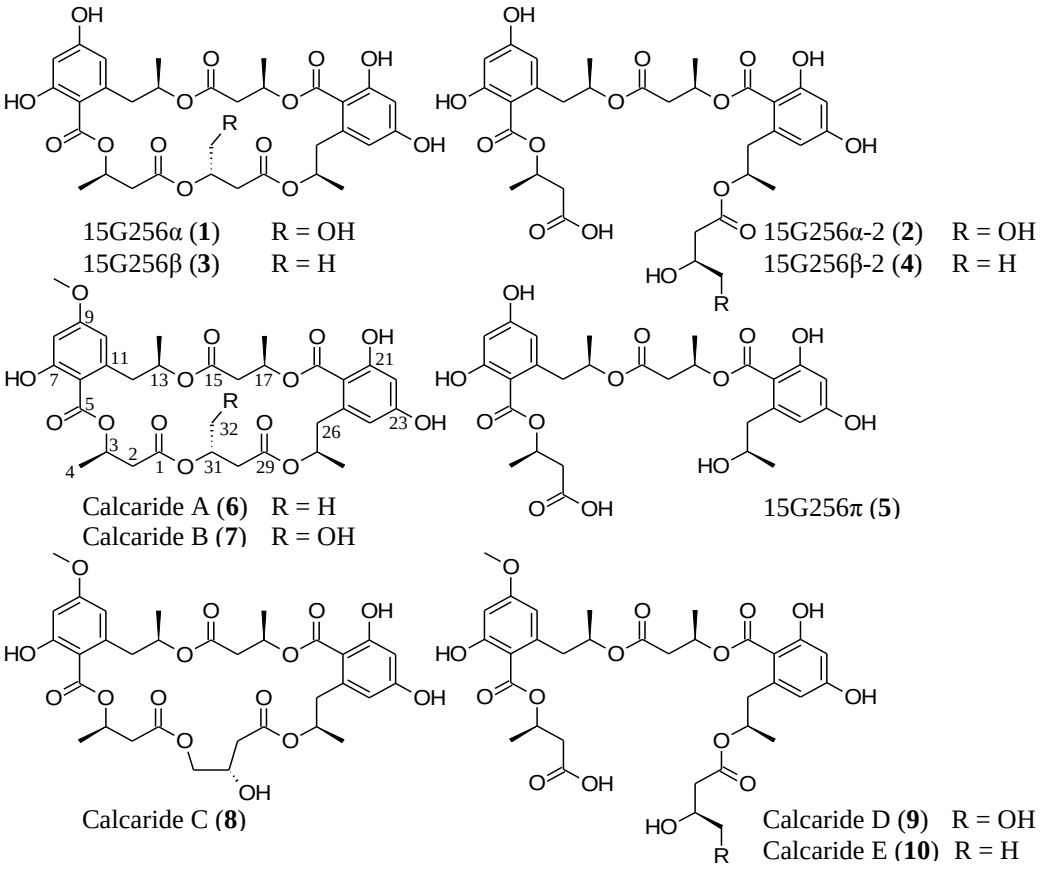

Figure II.1. Compounds isolated from *Calcarisporium* sp. KF525: 15G256α, α-2, β, β-2 and π (**1**–**5**) and calcarides A–E (**6**–**10**).

Here, we report the isolation of five known (**1**–**5**) and five new (**6**–**10**) 15G256-type compounds from *Calcarisporium* sp. strain KF525. Their structure elucidation and antibacterial activities are described.

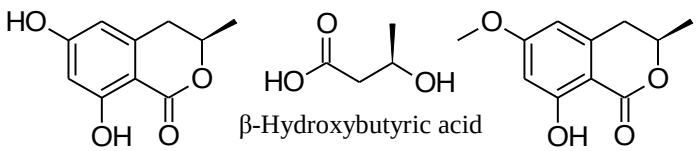

Figure II.2. Proposed biosynthetic precursors of the calcarides (based on the proposal of Schlingmann et al. 2002).

Results and Discussion

Isolation and Structure Elucidation

The fungal strain KF525 is a *Calcarisporium* sp. that is a part of the marine fungal strain collection of the Kiel Center for Marine Natural Products (Silber et al. 2013). Extracts of cultures from the *Calcarisporium* sp. strain KF525 grown in modified casamino acids glucose medium (Stevens 1974) showed antibacterial activities and were investigated in detail. Fractionation of these extracts by preparative reversed-phase HPLC yielded the known compounds, 15G256α, α-2, β, β-2 and π (**1**–**5**), as well as five new derivatives thereof, calcarides A–E (**6**–**10**) (Figure II.1).

The structures of **1**–**5** were identified by comparison of their spectroscopic data (UV, MS and ^{1}H NMR spectra) with those described in the literature (Schlingmann et al. 2002). As the data agreed well with the values published, the structures of **1**–**5** were assigned as shown in Figure II.1.

The molecular formula of calcaride A (**6**) was determined to be $C_{33}H_{40}O_{14}$ by high-resolution ESIMS measurements (measured 661.2503, calculated 661.2491 $[M + H]^{+}$). The structure of **6** was elucidated on the basis of one- and two-dimensional NMR spectra (^{1}H, ^{13}C, DEPT, COSY, HSQC and HMBC, see Table II.1). The NMR spectra of **6** were measured in acetone-d_6, as it did not dissolve well in methanol-d_4, whereas NMR data of all other calcarides were recorded in methanol-d_4. The analysis of the NMR data of **6** revealed considerable structural similarities with 15G256β (**3**). The ^{1}H NMR spectrum, the ^{1}H NMR coupling constants and the ^{13}C NMR spectrum of **6** showed that, comparable to **3** (Schlingmann et al. 2002, Breinholt et al. 1993), the compound was composed of two phenyl moieties with their two protons in *meta* position to each other and five methyl, five methylene, five methine and five ester carbonyl groups. The two-dimensional NMR spectra of **6** proved that the

aforementioned functional groups were connected exactly as in **3**, forming a cyclic pentalactone. A mass difference of 14 between **6** and **3** indicated the presence of an additional methyl group in **6**, which was corroborated by additional signals for a methyl group in the ^{1}H and ^{13}C NMR spectra. The chemical shifts of the respective proton and carbon resonances (δ_H 3.82 ppm, δ_C 55.8 ppm) determined it to be a methoxy group. The proton signal of the methoxy group correlated with C-9 in the HMBC NMR spectrum. Thus, the structure of **6** was identified as the methylated derivative of **3**, with the methyl group attached to the hydroxyl group of C-9.

The molecular formula of calcaride B (**7**) was deduced to be $C_{33}H_{40}O_{15}$ by high-resolution ESIMS measurements (measured 677.2435, calculated 677.2440 $[M + H]^+$). UV and NMR spectra of **7** (Tables II.2 and II.3) strongly resembled those of **6**. The NMR spectra of **7** showed all resonances as those of **6**, apart from one striking difference: the signals in position 32 corresponded to a methylene instead of a methyl group. The downfield shift of the proton and carbon signals of CH_2-32 (δ_H 3.65 and 3.58 ppm, δ_C 63.6 ppm) suggested the attachment of a hydroxyl group to C-32 in **7**. This was in full accordance with a mass increase of 16 mass units in comparison to **6** and determined **7** to be the methylated analog of the known compound, 15G256α (**1**).

Calcaride C (**8**) was found to have a molecular formula of $C_{33}H_{40}O_{15}$ by means of high-resolution ESIMS measurements (measured 677.2441, calculated 677.2440 $[M + H]^+$). Thus, **7** and **8** had the same molecular formula, showing that the closely related compounds were structural isomers, as was confirmed by comparison of the NMR spectra of the two compounds (Tables II.2 and II.3). For two of the known 15G256 components, 15G256α (**1**) and 15G256α-1 (Figure II.3), a comparable regioisomerism was observed in which either β- or γ-hydroxyester linkages lead to the ring closure of the compound (Schlingmann et al. 2002).

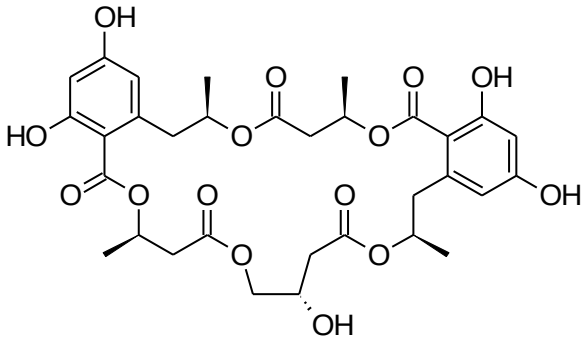

Figure II.3. Structure of 15G256 α-1 (Schlingmann et al. 2002).

Table II.1. NMR spectroscopic data (500 MHz, acetone-d_6) of calcaride A (**6**).

Position	δ_C, Type	δ_H, Mult. (*J* in Hz)	COSY	HMBC
1	170.09, C			
2a	40.6, CH_2	2.91, dd (6.7, 16.3)	2b, 3	1, 3, 4
2b		2.74, dd (6.4, 16.3)	2a, 3	1, 3, 4
3	70.1, CH[a]	5.56, m	2a, 2b, 4	1, 2, 4, 5
4	20.0, CH_3	1.450, d (6.4)	3	1, 2, 3
5	171.0, C			
6	106.9, C			
7	165.5, C			
8	100.4, CH	6.36, d (2.6)	10	5, 6, 7, 9, 10
9	164.8, C			
OCH_3	55.8, CH_3	3.82, s		9
10	111.9, CH	6.44, d (2.6)	8	5, 6, 8, 9, 12
11	142.9, C			
12a	41.7, CH_2	3.547, dd (6.8, 13.3)	12b, 13	6, 10, 11, 13, 14
12b		2.98, dd (7.5, 13.3)	12a, 13	6, 10, 11, 13, 14
13	73.1, CH	5.05, m[b]	12a, 12b, 14	11, 12, 14, 15
14	19.7, CH_3	1.19, d (6.2)	13	12, 13
15	170.12, C			
16a	41.02, CH_2	2.90, dd (6.3, 15.8)	16b, 17	15, 17, 18
16b		2.72, dd (7.1, 15.8)	16a, 17	15, 17, 18
17	70.0, CH[a]	5.52, m	16a, 16b, 18	15, 16, 18, 19
18	20.1, CH_3	1.454, d (6.4)	17	15, 16, 17
19	170.9, C			
20	105.6, C			
21	165.9, C			
22	102.6, CH	6.30, d (2.5)	24	19, 20, 21, 23,
23	163.1, C			
24	113.3, CH	6.35, d (2.5)	22	19, 20, 22, 23,
25	143.3, C			
26a	42.4, CH_2	3.553, dd (5.7, 12.9)	26b, 27	20, 24, 25, 27,
26b		2.78, dd (8.6, 12.9)	26a, 27	20, 24, 25, 27,
27	72.5, CH	5.01, m[b]	26a, 26b, 28	25, 26, 28, 29
28	19.5, CH_3	1.14, d (6.2)	27	26, 27
29	170.2, C			
30a	41.01, CH_2	2.63[c]	30b, 31	29, 31, 32
30b		2.63[c]	30a, 31	29, 31, 32
31	68.4, CH	5.28, m	30a, 30b, 32	1, 29, 30, 32
32	19.9, CH_3	1.27, d (6.3)	31	29, 30, 31

[a]Assignments of C-3 and C-17 are interchangeable. [b]Signals overlap and are deduced from the HSQC NMR spectrum. [c]Signals overlap.

Given that **7** is the methylated form of **1**, it was assumed that **8** was the methylated derivative of 15G256α-1. The NMR spectra of **8** confirmed the assumption, since the

signals were in good agreement with those reported for 15G256α-1 (Schlingmann et al. 2002). An additional resonance for a methoxy group in the NMR spectra of **8** determined it to be the methyl ether of 15G256α-1. As the assignments of C-7 (δ_C 165.08 ppm) and C-9 (δ_C 165.0 ppm) might be interchangeable, the position of the methoxy group that showed a correlation to either of the two signals in the HMBC spectrum could not unambiguously be determined. In analogy to **6**, **7**, **9** and **10**, where the methyl group was linked to the hydroxyl group of C-9, it was deduced that this is also most likely to be the case for compound **8**. In addition to being regioisomers, it was observed that **7** and **8** interconverted during storage as dried compounds, with **7** showing a purity of about 75% after a period of two weeks. At the same time, compound **8** had converted into **7** more slowly and showed a purity of around 85%. These percentages were stable over a period of three months in which the compound stability was studied.

The molecular formula of calcaride D (**9**) was determined as $C_{33}H_{42}O_{16}$ on the basis of high-resolution ESIMS measurements (measured 693.2405, calculated 693.2400 $[M - H]^-$), requiring 13 degrees of unsaturation. The NMR spectra of **9** (Tables II.3 and II.4) showed signals for the two phenolic substructures characteristic of the calcarides and five carbonyl resonances, together accounting for 13 double bond equivalents. These data suggested a ring opening for **9**, giving the structure of a linear polyester, as has been found among the 15G256 components, as well, e.g., 15G256α-2 (**2**), β-2 (**4**) and π (**5**). MS and NMR spectra proved **9** to be the methylated analog of **2**. For the linear calcarides (**9** and **10**), the NMR spectra recorded in methanol-d_4 gave one single signal for the aromatic protons of H-8 and H-10 (δ_H 6.33 ppm). Therefore, the position of the carbon signals of C-7 and C-9 (δ_C 166.0 and 165.0 ppm) could not be differentiated by the HMBC correlations of the aromatic protons resonances. As a consequence, the methoxy group showing a clear HMBC correlation to the signal at 165.0 ppm could have been attached to either C-7 or C-9. In order to determine the position of the methoxy group of **9**, additional NMR spectra were recorded in chloroform-d_1 in which signals of H-8 and H-10 appeared separately. The HMBC NMR spectrum displayed correlations of H-8 to C-7 and C-9 and of H-10 to C-9 and C-12. Thereby, the positions of the signals for C-7 and C-9 could be assigned. Consequently, the methoxy group for which the HMBC NMR spectrum displayed a correlation to the resonance of C-9 had to be attached to C-9, as shown in Figure II.1.

Table II.2. ^{1}H NMR spectroscopic data of calcarides A–C (**6**–**8**) (500 MHz, acetone-d_6 for **6**; 500 MHz, methanol-d_4 for **7**–**8**).

	Calcaride A (6)	**Calcaride B (7)**	**Calcaride C (8)**
Position	**δ_H, Mult. (*J* in Hz)**	**δ_H, Mult. (*J* in Hz)**	**δ_H, Mult. (*J* in Hz)**
2a	2.91, dd (6.7, 16.3)	2.88, dd (7.7, 16.6)	2.878, dd (8.1, 16.2)
2b	2.74, dd (6.4, 16.3)	2.73, d (5.3, 16.6)	2.79, dd (4.8, 16.2)
3	5.56, m	5.56, m	5.59, m
4	1.450, d (6.4)	1.42, d (6.3)	1.44, d (6.4)
8	6.36, d (2.6)	6.30, d (2.6)	6.31, d (2.6)
OCH_3	3.82, s	3.75, s	3.74, s[a]
10	6.44, d (2.6)	6.36, d (2.6)	6.35, d (2.6)
12a	3.547, dd (6.8, 13.3)	3.34[b]	3.35[c]
12b	2.98, dd (7.5, 13.3)	2.96, dd (6.4, 13.8)	2.99, dd (6.6, 14.0)
13	5.05, m[d]	5.07, m	5.16, m
14	1.19, d (6.2)	1.20, d (6.2)	1.22, d (6.2)
16a	2.90, dd (6.3, 15.8)	2.81, dd (7.6, 15.8)	2.85, dd (6.6, 16.0)
16b	2.72, dd (7.1, 15.8)	2.67, dd (5.7, 15.8)	2.69, dd (6.6, 16.0)
17	5.52, m	5.49, m	5.48, m
18	1.454, d (6.4)	1.40, d (6.4)	1.39, d (6.3)
22	6.30, d (2.5)	6.22, s	6.21, d (2.5)
24	6.35, d (2.5)	6.22, s	6.23, d (2.5)
26a	3.553, dd (5.7, 12.9)	3.32[b]	3.31[c]
26b	2.78, dd (8.6, 12.9)	2.77, dd (8.1, 13.3)	2.885, dd (6.9, 13.4)
27	5.01, m[d]	4.98, m	5.03, m
28	1.14, d (6.2)	1.11, d (6.2)	1.16, d (6.2)
30a	2.63[e]	2.68, dd (5.1, 16.7)	2.51, dd (6.0, 15.3)
30b	2.63[e]	2.64, dd (8.1, 16.7)	2.43, dd (7.2, 15.3)
31	5.28, m	5.28, m	4.17, m
32a	1.27, d (6.3)	3.65, dd (4.5, 11.9)	4.12, dd (4.8, 11.1)
32b		3.58, dd (5.1, 11.9)	4.08, dd (5.5, 11.1)

[a]Position of the methoxy group could not unambiguously be determined. In analogy to the other calcarides, it was assumed to be linked to C-9. [b]Signal partially obscured and deduced from the HMBC NMR spectrum. [c]Signal partially obscured. [d]Signals overlap and are deduced from the HSQC NMR spectrum. [e]Signals overlap.

The molecular formula of calcaride E (**10**) was determined to be $C_{33}H_{42}O_{15}$ by high-resolution ESIMS measurements (measured 677.2467, calculated 677.2451 $[M - H]^-$). The structure of **10** was readily deduced by comparing the NMR data (Tables II.3 and II.4) with those of **9**. In contrast to the hydroxylated methylene group at C-32 in **9**, the corresponding signals in the NMR spectra of **10** gave evidence for a methyl group not adjacent to oxygen. The position of the methoxy group at C-9 was determined according to additionally recorded NMR spectra in chloroform-d_1, as described for **9**.

Hence, the structure of **10** was established as the dehydroxylated form of **9**, which could equivalently be described as the methylated analog of **4**.

Table II.3. ^{13}C NMR shifts of calcarides A–E (**6**–**10**) (125 MHz, acetone-d_6 for **6**; 125 MHz, methanol-d_4 for **7**–**9**; 150 MHz, methanol-d_4 for **10**).

	Calcaride A (6)	**Calcaride B (7)**	**Calcaride C (8)**	**Calcaride D (9)**	**Calcaride E (10)**
Position	**δ_C**	**δ_C**	**δ_C**	**δ_C**	**δ_C**
1	170.09	171.5[a]	171.6	173.9[b]	175.9[b]
2	40.6	41.0	41.2	41.8	43.3
3	70.1[c]	70.2	70.5	70.8	71.5
4	20.0	20.2	20.2	20.1	20.3
5	171.0	171.13	171.53[d]	171.5[e]	171.56[f]
6	106.9	108.7	108.2	107.2	107.3
7	165.5	164.6	165.08[g,h]	166.0[i]	166.0[j]
8	100.4	100.7	100.7	100.8	100.8
9	164.8	164.9	165.0[h]	165.0[i]	165.0[j]
OCH_3	55.8	55.8	55.8[k]	55.9	55.9
10	111.9	111.5	111.3	112.8	112.8
11	142.9	142.6	142.7	143.2	143.3
12	41.7	41.8	41.7	43.2	43.4
13	73.1	73.6	73.2	73.1	73.2
14	19.7	20.0	20.0	20.52	20.6
15	170.12	171.37[a]	171.3[d]	171.2	171.2
16	41.02	41.6	41.6[l]	41.8	41.8
17	70.0[c]	70.4	70.3	70.0	70.0
18	20.1	20.2	20.1	19.9	19.9
19	170.9	171.07	171.48[d]	171.6[e]	171.62[f]
20	105.6	106.7	107.3	105.8	105.7
21	165.9	165.4	165.14[g]	166.2	166.3
22	102.6	102.8	102.7	102.7	102.7
23	163.1	163.5	163.4	163.5	163.6
24	113.3	113.3	112.6	113.8	113.9
25	143.3	143.2	143.1	143.7	143.7
26	42.4	42.3	41.5[l]	43.5	43.6
27	72.5	73.5	73.6	72.7	72.6
28	19.5	19.5	19.8	20.49	20.6
29	170.2	171.43[a]	171.9	172.7	172.5
30	41.01	36.7	40.4	39.9	45.1
31	68.4	72.7	67.1	69.9	65.4
32	19.9	63.6	68.5	66.4	23.0

[a,c–j,l]Assignments are interchangeable. [b]Signal deduced from the HMBC NMR spectrum. [k]Position of the methoxy group could not unambiguously be determined. In analogy to the other calcarides it was assumed to be linked to C-9.

Table II.4. [1]H NMR spectroscopic data of calcarides D and E (**9** and **10**) (500 MHz, methanol-d_4 for **9**; 600 MHZ, methanol-d_4 for **10**).

	Calcaride D (9)	**Calcaride E (10)**
Position	**δ_H, Mult. (*J* in Hz)**	**δ_H, Mult. (*J* in Hz)**
2a	2.767, dd (7.5, 15.8)	2.72, dd (7.8, 15.4)
2b	2.69, dd (5.3, 15.8)	2.61, dd (5.9, 15.4)[a]
3	5.57, m	5.58, m
4	1.43, d (6.4)	1.43, d (6.3)
8	6.33, s	6.33, s
OCH_3	3.77, s	3.77, s
10	6.33, s	6.33, s
12a	3.29[b]	3.35, dd (3.5, 13.6)[a]
12b	2.91, dd (9.7, 13.7)	2.87, dd (9.8, 13.6)
13	5.22, m	5.22, m
14	1.25, d (6.2)	1.26, d (6.3)
16a	2.66, dd (7.2, 15.7)	2.65, dd (7.2, 15.6)
16b	2.62, dd (5.8, 15.7)	2.62, dd (6.0, 15.6)
17	5.47, m	5.47, m
18	1.30, d (6.4)	1.30, d (6.3)
22	6.203, d (2.6)	6.19, d (2.5)
24	6.197, d (2.6)	6.18, d (2.5)
26a	3.17, dd (4.0, 13.4)	3.20, dd (3.8, 13.5)
26b	2.772, dd (9.2, 13.4)	2.74, dd (9.5, 13.5)
27	5.15, m	5.14, m
28	1.23, d (6.2)	1.23, d (6.2)
30a	2.47, dd (5.2, 15.4)	2.36, dd (6.9, 14.7)
30b	2.27, dd (7.9, 15.4)	2.26, dd (6.5, 14.7)
31	3.94, m	4.02, m
32a	3.44, dd (4.9, 11.2)	1.05, d (6.2)
32b	3.40, dd (5.7, 11.2)	

[a]Signal partially obscured. [b]Signal partially obscured and is deduced from the HSQC NMR spectrum.

Because **6**–**10** were methylated forms of known compounds, it had to be ruled out that they were artifacts arising from the extraction protocol employing methanol as the solvent. The first indication that this was not the case for **6**–**10** was that the four demethylated analogs **1**–**4**, which were, as well, isolated from *Calcarisporium* sp. KF525, stayed demethylated when stored as methanolic solutions for time periods of more than two weeks. The final proof was that the isolation of **6**–**10** was likewise possible, when the use of methanol was avoided in all extraction steps and acetonitrile was employed instead. For this purpose, the presence of **6**–**10** was confirmed by using HPLC-UV/MS fingerprints of extracts from methanol and acetonitrile.

Absolute configurations of **6**–**10** were investigated by measurements of the optical rotation. The obtained values for optical activity were compared to those described for the demethylated analogs. The optical rotation data of **6**–**8** ($[\alpha]^{22}_{D}$ −23.6, −25.3 and −7.9, respectively) were in good agreement with the reported ones of the corresponding demethylated forms, **3**, **1** and 15G256α-1 (Schlingmann et al. 2002, Breinholt et al. 1993, Ito et al. 1992a, Roy et al. 1996). Even though some of the published values referred to ethanol solutions or higher temperatures employed for the measurements, they were consistent with our data. Hence, the absolute configurations of **6**–**8** were suggested to be the same as for their published demethylated forms (Schlingmann et al. 2002). For **2** and **4**, which are the demethylated derivatives of **9** and **10**, there is no literature record of optical rotation values. Thus, a comparison with published data was not possible, but as **2** and **4** were also isolated from *Calcarisporium* sp. KF525, their optical rotation was measured ($[\alpha]^{22}_{D}$ −77.8 for **2**; $[\alpha]^{22}_{D}$ −76.9 for **4**) and compared with the values of **9** and **10** ($[\alpha]^{22}_{D}$ −71.8 and −67.3, respectively). As a result, the stereochemistry of **9** and **10** was assumed to be identical to the stereochemistry for **2** and **4** proposed by Schlingmann et al. (2002) (Figure II.1).

From a biosynthetic perspective, the precursors of the known 15G256 components are 6-hydroxymellein and β-hydroxybutyric acid (Schlingmann et al. 2002). Consequently, the building blocks of the calcarides should be 6-methoxymellein additional to 6-hydroxymellein and β-hydroxybutyric acid (Figure II.2). 6-methoxymellein is a natural product known from a variety of fungi, e.g., *Aspergillus* and *Ceratocystis* species (Dictionary of Natural Products 2012). Both, 6-methoxy- and 6-hydroxy-mellein were found in crude extracts of *Calcarisporium* sp. KF525, according to UV and MS spectra from analytical HPLC-UV/MS analyses. The position of the methoxy and hydroxyl groups in the mellein structure could not be deduced from these data and was only assumed according to the position of the respective groups in the calcarides. Given that the calcarides are built up by the same or methylated precursors as the 15G256 components, the identical stereochemistry in calcaride and 15G256 molecules is reasonable.

Antibacterial Activity

Compounds **1**–**10** were profiled against a set of bacterial strains, including the biofilm-forming, *Staphylococcus epidermidis*, the plant pathogen, *Xanthomonas campestris*, and *Propionibacterium acnes*, which is linked to the skin disease acne. Even though the

compounds are closely related from a structural point of view, they show distinct antibacterial activities (Table II.5). All macrocyclic compounds (**1**, **3**, **6**–**8**) inhibited *S. epidermidis* and *X. campestris*, while the linear polyesters did not show any activities below a minimal inhibitory concentration (MIC) of 100 µM. The results indicated that a ring structure is required for the activity. This observation was also made for antifungal properties of the already known 15G256 components (Schlingmann et al. 2002). Among the cyclic compounds, **1** showed the highest activity against *S. epidermidis* ((12.9 ± 3.6) µM), and **6** displayed the lowest MIC for *X. campestris* ((5.5 ± 1.3) µM). A comparison of the inhibition between active methylated and demethylated forms, e.g., **1** and **7** or **3** and **6**, showed a decreased activity against *S. epidermidis* by a factor of four for the methylated analogs, whereas the activities concerning *X. campestris* were in the same concentration range. *P. acnes* was not inhibited by any of the tested substances, but the linear compound **5**. Given that **5** and **4** only differ in a β-hydroxybutyrate moiety, the *P. acnes*-related activity of **5** seemed highly specific.

Table II.5. Antibacterial minimal inhibitory concentrations (MICs) of compounds **1**–**10** and the positive control, chloramphenicol. Activities higher than 20% were considered as inhibitory.

Compound	***S. epidermidis*** **MIC [µM]**	***X. campestris*** **MIC [µM]**	***P. acnes*** **MIC [µM]**
15G256α (**1**)	12.9 (±3.6)	30.8 (±3.1)	>100
15G256α-2 (**2**)	>150	>150	>200
15G256β (**3**)	16.9 (±0.6)	14.9 (±7.5)	>200
16G256β-2 (**4**)	>150	>150	>100
15G256π (**5**)	>150	>150	14.1 (±1.8)
calcaride A (**6**)	68.8 (±3.7)	5.5 (±1.3)	>200
calcaride B (**7**)	52.3 (±2.3)	22.6 (±9.2)	>200
calcaride C (**8**)	29.6 (±1.6)	61.4 (±12.7)	>100
calcaride D (**9**)	>150	>150	>200
calcaride E (**10**)	104.3 (±7.8)	>150	>100
chloramphenicol	4.5	1.0	0.5

15G256 components are known to possess antifungal activities, estrogenic effects or cytotoxic properties with regard to cellular ATP (viability) and chemiluminescence (phagocytosis) in a mouse peritoneal macrophage assay, as well as they are described to be potentiators of the nerve growth factor in PC12 cells (Schlingmann et al. 2002, Breinholt et al. 1993, Ito et al. 1992a, Roy et al. 1996, Kondo el. 1993). However, no antibacterial activities had been observed, even when tested. Here, the 15G256 compounds and the new methylated derivatives thereof were found to display antibacterial activities that are moderate in potency, but have a tight structure-activity relationship. Even a slight structural variation, like a methylation, as seen for the calcarides, influenced compound activities significantly. This was found to be true for other natural products (Walle et al. 2007, Liu et al. 2010). For instance, the methylation of the cholesterol-lowering agent, lovastatin, resulted in the improved drug, simvastatin (Li and Vederas 2009). Regarding simvastatin, the introduction of a methyl group was used as a tool, but similarly, the isolation of derivatives of known compounds can be used as a strategy to obtain improved lead structures. As shown for the calcarides, fungi are a good source for this purpose, because they often produce a bunch of structurally related compounds, e.g., resulting from the promiscuity of the biosynthetic enzymes (Grüschow et al. 2011). From the fungus' point of view, the production of closely-related components might be of advantage as altered activities emerge.

Experimental Section

General Experimental Procedures

Optical rotation measurements were obtained on a Perkin Elmer model 241 polarimeter. UV spectra were measured on a Perkin Elmer Lambda 2 photometer. NMR spectra were recorded on a Bruker DRX 500 (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively) and a Bruker AV 600 spectrometer (600 and 150 MHz for ^{1}H and ^{13}C NMR, respectively). The residual solvent signals were used as internal references for NMR analyses (δ_H 3.31 and δ_C 49.0 ppm for methanol-d_4; δ_H 2.05 and δ_C 29.8 ppm for acetone-d_6, δ_H 7.26 and δ_C 77.2 ppm for chloroform-d_1). High-resolution mass spectra were measured with a Bruker micrOTOF II, applying positive or negative ESI mode. Analytical reversed-phase HPLC-UV/MS experiments were conducted on a VWR-Hitachi LaChrom Elite system equipped with an L-2130 pump, an L-2450 diode array detector, an L-2200 autosampler, an L-2300 column oven and a

Phenomenex Onyx Monolithic column (C18, 100 × 3.00 mm). A gradient with 0.1% formic acid in H_2O (A) and 0.1% formic acid in acetonitrile (B) as solvents was applied: 0 min 5% B, 4 min 60% B, 6 min 100% B; flow 2 mL min^{-1}. For mass detection, the analytical HPLC system was coupled to a Bruker esquire4000 ion-trap detector. Preparative HPLC was performed on a VWR LaPrep system consisting of a P110 pump, a P311 UV detector, a Labocol Vario-2000 fraction collector (LABOMATIC), a Smartline 3900 autosampler (Knauer) and a Phenomenex Gemini-NX column (10 µ C18, 100A, Axia, 100 × 50.00 mm). Subsequent compound purifications were performed on a Merck-Hitachi LaChrom Elite HPLC system comprised of an L-7150 pump, an L-2450 diode array detector and an L-2200 autosampler employing a reversed-phase Phenomenex Gemini-NX column (5 µ C18, 110A, Axia, 100 × 21.20 mm). 0.1% of formic acid in H_2O (A) and 0.1% formic acid in acetonitrile (B) were the solvents used in preparative HPLC separations.

Fungal Material

Calcarisporium sp. KF525 is a part of the marine fungal strain collection of the Kiel Center for Marine Natural Products. The strain was cryo-conserved using the Microbank system (Pro-Lab).

Cultivation

Calcarisporium sp. KF525 was cultivated in 12 L of casamino acids glucose medium (0.25% casein hydrolysate, 4% glucose × H_2O, 0.01% $MgSO_4$ × $7H_2O$, 0.18% KH_2PO_4, pH 6.8) (Stevens 1974). 2-L Erlenmeyer flasks filled with 0.75 L medium were used for the culture experiments, which were performed for 24 days at 22 °C under shaking conditions (120 rpm) in the dark. A circular agar slant (1.8 cm in diameter) of a preculture served as the inoculum. The preculture was grown on agar plates of modified Wickerham medium (0.3% malt extract, 0.3% yeast extract, 0.5% peptone from soymeal, 1% glucose × H_2O, 3% NaCl, 1.5% agar, pH 6.25) (Wickerham 1951) over a period of 11 days at room temperature in the dark.

Isolation Procedure

The cultures of *Calcarisporium* sp. KF525 were separated into mycelium and culture supernatant. An extraction of the culture supernatant with EtOAc yielded a crude extract of 0.92 g. The extract was subjected to the first purification step using a VWR LaPrep

HPLC system (gradient: 0 min 10% B, 17 min 60% B, 22 min 100% B; flow 100 mL min^{-1}; UV detection at 217 nm). The purification gave 8 fractions containing the 15G256 and calcaride components (t_R 12.4, 13.2, 14.5, 15.7, 16.2, 17.6, 18.5 and 20.6 min) of which fraction 8 comprised pure **6** in a yield of 17.7 mg. All other fractions were further purified on a Merck-Hitachi LaChrom Elite HPLC system applying reversed-phase chromatography. The purification of fraction 1 gave 17.8 mg of **2** (gradient: 0 min 30% B, 13 min 40% B; flow 18 mL min^{-1}; UV detection at 220 nm; t_R 6.7 min) and fraction 2 afforded 11.8 mg of **5** (gradient: 0 min 25% B, 13 min 50% B; flow 18 mL min^{-1}; UV detection at 220 nm; t_R 9.2 min). The chromatography of fraction 3 (gradient: 0 min 30% B, 15 min 37% B; flow 18 mL min^{-1}; UV detection at 220 nm) resulted in 14 mg of purified **4** (t_R 11.1 min) and semi-pure **9** (t_R 12.0 min), which was isolated in two additional rounds of purification to give a yield of 23.2 mg (1st, gradient: 0 min 30% B, 13 min 45% B; flow 18 mL min^{-1}; UV detection at 220 nm; t_R 9.2 min. 2nd, isocratic: 25% B; flow 18 mL min^{-1}; UV detection at 210 nm; t_R 23.2 min). Pure **1** (17.5 mg) was obtained from fraction 4 by applying two consecutive steps of preparative HPLC (1st, 0 min 40% B, 13 min 65% B, flow 18 mL min^{-1}; UV detection at 205 nm; t_R 5.2 min. 2nd, gradient: 0 min 30% B, 10 min 61% B; flow 18 mL min^{-1}; UV detection at 220 nm; t_R 7.6 min). Fraction 5 was subjected to two further purification steps yielding 2.9 mg of **10** (1st, isocratic: 29% B, flow 18 mL min^{-1}; UV detection at 220 nm; t_R 21.7 min. 2nd, gradient: 0 min 27% B, 15 min 50% B; flow 18 mL min^{-1}; UV detection at 220 nm; t_R 12.4 min). Fraction 6 contained compounds **7** and **8**, which were initially eluted isocratically with 42% B (flow 18 mL min^{-1}; UV detection at 220 nm; t_R 11.8 min). A subsequent round of preparative HPLC (isocratic: 40%, flow 18 mL min^{-1}; UV detection at 210 nm) gave purified **7** (t_R 12.8 min) and **8** (t_R 16.0 min) in yields of 14.6 and 5.7 mg, respectively. 1.8 mg of compound **3** were isolated from fraction 7 by employing two chromatographic steps (1st, gradient: 0 min 50% B, 13 min 80% B; flow 18 mL min^{-1}; UV detection at 220 nm; t_R 6.0 min. 2nd, isocratic: 45% B, flow 18 mL min^{-1}; UV detection at 220 nm; t_R 7.3 min.).

Calcaride A (6): Pale yellow solid, $[\alpha]^{22}_D$ −23.6 (*c* 0.195, MeOH), UV (MeOH) λ_{max} (log ε) 216 (4.57), 263 (4.27), 302 (3.92) nm; for 1D and 2D NMR data see Table II.1; HRESIMS *m/z* 661.2503 $[M + H]^+$ (calcd for $C_{33}H_{40}O_{14}$, 661.2491).

Calcaride B (7): White solid, $[\alpha]^{22}_{D}$ −25.3 (*c* 0.45, MeOH), UV (MeOH) λ_{max} (log ε) 216 (4.58), 263 (4.27), 302 (3.92) nm; for 1D and 2D NMR data see Tables II.2, II.3 and SI; HRESIMS *m/z* 677.2435 $[M + H]^+$ (calcd for $C_{33}H_{40}O_{15}$, 677.2440).

Calcaride C (8): White solid, $[\alpha]^{22}_{D}$ −7.9 (*c* 0.793, MeOH), UV (MeOH) λ_{max} (log ε) 216 (4.59), 263 (4.30), 302 (3.94) nm; for 1D and 2D NMR data see Tables II.2, II.3 and SI; HRESIMS *m/z* 677.2441 $[M + H]^+$ (calcd for $C_{33}H_{40}O_{15}$, 677.2440).

Calcaride D (9): White solid, $[\alpha]^{22}_{D}$ −71.8 (*c* 0.98, MeOH), UV (MeOH) λ_{max} (log ε) 216 (4.60), 263 (4.35), 302 (3.99) nm; for 1D and 2D NMR data see Tables II.3, II.4 and SI; HRESIMS *m/z* 693.2405 $[M - H]^-$ (calcd for $C_{33}H_{42}O_{16}$, 693.2400).

Calcaride E (10): White solid, $[\alpha]^{22}_{D}$ −67.3 (*c* 0.05, MeOH), UV (MeOH) λ_{max} (log ε) 216 (4.61), 263 (4.35), 302 (3.98) nm; for 1D and 2D NMR data see Tables II.3, II.4 and SI; HRESIMS *m/z* 677.2467 $[M - H]^-$ (calcd for $C_{33}H_{42}O_{15}$, 677.2451).

The process of interconversion of **7** and **8** was studied by periodical HPLC-UV/MS analyses using the mass chromatograms for quantification of the compounds' purities. For the experiments, purified and dried **7** and **8** were stored at 4 °C over a time period of three months.

Antibacterial Assays

Antibacterial assays were performed using the test strains, *Staphylococcus epidermidis* (DSM20044), *Xanthomonas campestris* (DSM 2405) and *Propionibacterium acnes* (DSM1897).

S. epidermidis and *X. campestris* were cultivated in trypticase soy broth (1.2% trypticase soy broth, 0.5% NaCl) overnight and diluted to an OD_{600} of 0.01–0.03. 10 mM DMSO solutions of the compounds were diluted with medium to gain desired test concentrations. 10.5 µL of the compound solutions and 200 µL of the respective test strain cell suspension were transferred into a 96-well microtiter plate. Microtiter plates with *S. epidermidis* were incubated for 5 h at 37 °C in the dark, while microtiter plates with *X. campestris* were incubated for 6 h at 28 °C in the dark. After the addition of 10 µL of a resazurin solution as the detective reagent (0.2 mg mL^{-1} in phosphate-buffered saline), the incubation was continued for another 5–30 min. To evaluate cell viability, the reduction of resazurin to resorufin was determined by measuring the intensity of fluorescence at $560_{Ex}/590_{Em}$ in a Tecan Infinite M200 plate reader.

P. acnes was grown anaerobically (Anaerocult A mini, Merck) in modified PYG medium (DSMZ medium 104) for 48 h at 37 °C in the dark. 2-day old cultures were diluted to an OD_{600} of 0.02. A volume of 200 µL of the cell suspension was transferred to a microtiter plate and mixed with 10.5 µL of the test compounds that were treated as described above. After an anaerobic incubation for 48 h at 37 °C in the dark, 10 µL of bromocresol purple (1 mg mL^{-1}) were added, and the acid production of *P. acnes* was determined by measuring the absorbance at 590 nm (reference 690 nm) using the Infinite M200 plate reader.

The resulting values of the antibacterial assays were compared with a positive control (10 µM chloramphenicol for *S. epidermidis* or *X. campestris* and 1 µM chloramphenicol for *P. acnes*) and a negative control (no compound). Minimal inhibitory concentrations (MICs) were defined as more than 20% inhibition of metabolic activity or acid production in the test strains compared to non-inhibited metabolism in the negative control.

Conclusions

Five known 15G256 components and five methylated analogs thereof were isolated from *Calcarisporium* sp. strain KF525: 15G256α, α-2, β, β-2, π (**1**–**5**) and calcarides A–E (**6**–**10**). For the first time, antibacterial properties were detected for this type of natural product. The compounds showed inhibitory bioactivities against *Staphylococcus epidermidis*, *Xanthomonas campestris* and *Propionibacterium acnes*, underlying a tight structure-activity relationship. 15G256α (**1**) displayed the strongest inhibition against *S. epidermidis*, with a MIC of (12.9 ± 3.6) µM, and calcaride A (**6**) exhibited the lowest MIC for *X. campestris* with (5.5 ± 1.3) µM. *P. acnes* was specifically inhibited by the compound, 15G256π (**5**), having a MIC of (14.1 ± 1.8) µM.

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CHAPTER III

Malettinin E, antimicrobial tropolone produced by a *Cladosporium* strain

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to be submitted

Abstract

The isolation and structure elucidation of malettinins A–C (**1**–**3**) along with the new malettinin E (**4**) are described. The compounds were produced by the fungus *Cladosporium* sp. strain KF501, which was isolated from the German Wadden Sea. The malettinins are built up of tropolone/dihydropyran ring structures linked to a furan ring. The structure elucidation of the isolated compounds was achieved by means of one- and two-dimensional NMR spectroscopy supported by mass and UV data. The relative configuration of **4** was determined on the basis of single-crystal X-ray diffraction analysis. **1**–**4** exhibited antibacterial and antifungal activities when profiled against *Xanthomonas campestris* and *Trichophyton rubrum*. The influence of the chemical structure of the furan ring and of configurational changes on biological activities was observed.

Introduction

The genus *Cladosporium* represents one of the largest and most heterogeneous genera of the Hyphomycetes (Bensch et al. 2012), showing ubiquitous occurrence. Species of the genus often have pathogenic or saprophytic lifestyles and are frequently found in air, soil, on foods, on plant materials or as endophytes (Stevens 1974, Samson et al. 2000). Occurring as airborne fungi, some species of *Cladosporium* are of clinical importance as they can cause allergies such as allergic asthma (Simon-Nobbe et al. 2008). Concerning marine habitats, a minimum of three *Cladosporium* species were described from marine sources (Kirk and Clipson 2013). *Cladosporium* species have been shown to possess the ability to produce a variety of natural products, among them the melanins which are pigments giving the fungal colonies their typical dark colored appearance. Other natural products isolated from *Cladosporium* species are bioactive compounds such as the antifungal cladosporides (Hosoe et al. 2000, Hosoe et al. 2001), the plant growth factors cotylenins (Sassa 1971, Sassa et al. 1975), calphostins which specifically inhibit the protein kinase C (Kobayashi et al. 1989) and cladosporin exhibiting a broad activity spectrum including antifungal, antibacterial, insecticidal, phytotoxic and immunosuppressive properties (Scott et al. 1971, Anke et al. 1978, Grove and Pople 1981, Springer et al. 1981, Fujimoto et al. 1999).

In this study, a marine-derived *Cladosporium* strain was shown to produce the new compound malettinin E (**4**) along with the known malettinins A–C (**1**–**3**) (Figure III.1),

which have not been isolated from this genus before. The structure elucidation of the compounds and their biological activities are described.

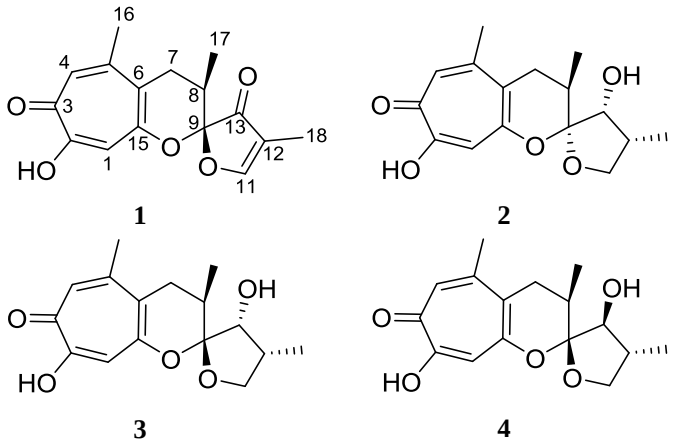

Figure III.1. Structures of malettinins A–C (**1**–**3**) and malettinin E (**4**), isolated from *Cladosporium* sp. strain KF501.

Material and Methods

General Experimental Procedures

Melting points were determined on an Electrothermal melting point apparatus. Measurements of optical rotation were performed on a Perkin Elmer model 241 polarimeter. UV spectra were recorded on a Perkin Elmer Lambda 2 spectrophotometer. NMR spectra were measured on a Bruker AV 600 spectrometer (600 and 150 MHz for ^{1}H and ^{13}C NMR, respectively) and the residual solvent signals served as internal references (δ_H 3.31 and δ_C 49.0 for methanol-d_4). High-resolution mass spectra were obtained on a Bruker micrOTOF II spectrometer using ESI ion source in negative mode. Analytical HPLC-UV/MS was conducted on a VWR-Hitachi LaChrom Elite system (pump L-2130, diode array detector L-2450, autosampler L-2200 and column oven L-2300) with a Phenomenex Onyx Monolithic column (C18, 100 × 3.00 mm) applying a gradient of 0.1% formic acid in H_2O (A) and 0.1% formic acid in acetonitrile (B): 0 min 5% B, 4 min 60% B, 6 min 100% B; flow 2 mL min^{-1}. Coupling of the HPLC system to a Bruker esquire4000 ESI-ion trap allowed mass detection.

Isolation and Taxonomy of Producing Organism

Strain KF501 was isolated from a water sample taken in the German Wadden Sea. DNA extraction, amplification of the internal transcribed spacer region and sequencing were

performed as described by Wiese et al. (2011) with slight modifications, centrifugation of the DNA at 8000*g* and 35 cycles of DNA amplification. The DNA sequence was deposited in GenBank under the accession number KF923800. Cryo-conserved stock cultures of strain KF501 were kept at -100 °C using the Microbank system (Pro-Lab).

Fermentation and Compound Isolation

The fungal isolate was cultivated in the following media: Casamino acids glucose medium (casein hydrolysate 0.25%, glucose × H_2O 4%, $MgSO_4$ × $7H_2O$ 0.01%, KH_2PO_4 0.18%, pH 6.8) (Stevens 1974), Czapek medium (sucrose 3%, $NaNO_3$ 0.3%, K_2HPO_4 0.1%, KCl 0.05%, $MgSO_4$ × $7H_2O$ 0.05%, $FeSO_4$ × $7H_2O$ 0.001%, pH 6.2) (Samson et al. 2000), modified malt extract medium (malt extract 3%, NaCl 1.5%, pH 5.5) (Samson et al. 2000), potato-carrot medium (potatoes 40 g, carrots 40 g, each boiled in 1 L of H_2O and filtered off, 250 mL of potato extract and 250 mL of carrot extract were filled up with 500 mL of distilled H_2O) (Samson et al. 2000) and modified Wickerham medium (malt extract 0.3%, yeast extract 0.3%, peptone from soymeal 0.5%, glucose × H_2O 1%, NaCl 3%, agar 1.5%, pH 6.25) (Wickerham 1951).

For isolation of the malettinins, *Cladosporium* sp. KF501 was cultivated in 9.75 L of casamino acids glucose medium. The cultivation experiments were performed as surface cultures at 20 °C in the dark in 2-L Erlenmeyer flasks, each containing 750 mL medium. The cultures were inoculated with an agar slant (2.6 cm in diameter) of a seven days old preculture, which was grown on solid, modified Wickerham medium at room temperature in the dark. The mycelium of strain KF501 was harvested after 40 days of incubation by extraction with EtOH. As the obtained extract was very greasy, fats were frozen out at -20 °C yielding 1.2 g of extract.

The malettinins were purified from the mycelium extract by preparative HPLC. The separations were performed on a VWR LaPrep system (pump P110, UV detector P311 UV, fraction collector Labocol Vario-2000 from LABOMATIC, autosampler Smartline 3900 from Knauer) using a Phenomenex Gemini-NX column (10 µ C18, 100A, Axia, 100 × 50.00 mm). A gradient of 0.1% formic acid in H_2O (A) and 0.1% formic acid in acetonitrile (B) (0.0 min 20% B, 17.5 min 40% B, 21.0 min 60% B; flow 100 mL min^{-1}) was applied to yield 17.5 mg of **2** (t_R 8.6-9.0 min), 6.1 mg of **3** (t_R 9.2-9.6 min), 4.6 mg of **4** (t_R 9.9-10.3 min) and 15.1 mg of **1** (t_R 12.2-18.0 min).

X-ray Crystal Structure Determination

Single crystals of **4** suitable for X-ray data collection were obtained by recrystallization from a methanol solution. The data were measured using an Imaging Plate Diffraction System (IPDS-2) from STOE & CIE (Table III.1). All non-hydrogen atoms were refined anisotropically. The H atoms were positioned with idealized geometry (O-H H atoms allowed to rotate but not to tip) and refined isotropically with $U_{iso}(H) = 1.2 \cdot U_{eq}(C)$ (1.5 for methyl and O-H H atoms) using a riding model. There are two crystallographically independent molecules in the asymmetric unit, which exhibit the same relative configuration. In one of these molecules the H atoms of one methyl group are disordered and were refined using a split model with two orientations rotated by 60°. Because no strong anomalous scattering atoms are present, the absolute configuration cannot be determined. Therefore, Friedel opposites were merged in the refinement.

Table III.1. Selected crystal data and details of the structure refinement for **4**.

Compound	**4**
Formula	$C_{16}H_{20}O_5$
MW / g mol^{-1}	292.32
Crystal system	triclinic
Space group	*P*1
a /Å	5.6652(6)
b / Å	10.7203(10)
c / Å	12.0282(12)
α / °	101.951(8)
β / °	97.120(8)
γ / °	90.126
V / Å^3	708.87(12)
T / K	150
Z	2
D_{calc} / mg m^3	1.370
μ / mm^{-1}	0.101
θ_{max} / °	24.99
Refl. collected	6705
Unique refl.	2486
R_{int}	0.1231
Refl. [F_0>4σ(F_0)]	1795
Parameters	384
R_1 [F_0>4σ(F_0)]	0.0687
wR_2 (all data)	0.1874
GOF	1.081
$\Delta\rho_{max/min}$ / e Å^{-3}	0.203 / -0-207

Antimicrobial Assays

Antimicrobial assays with the test strains *Xanthomonas campestris* and *Staphylococcus epidermidis* were performed as described by Silber et al. (2013). *Bacillus subtilis* and *Candida albicans* were tested as described by Ohlendorf et al. (2012). For antifungal assays using *Trichophyton rubrum*, cultivation was carried out in modified Sabouraud medium (peptone 1%, glucose 2%, pH 5.6). A spore solution containing 5×10^4 spores of *T. rubrum* per mL medium was prepared. 10 mM DMSO solutions of compounds were diluted with medium to achieve the desired test concentrations. For assaying, 200 µL of the spore solutions were mixed with 10.5 µL of compound solutions in 96-well microtiter plates and incubated for 72 h at 28 °C in the dark. The cell growth of *T. rubrum* was evaluated by measuring the optical densities with a Tecan Infinite M200 plate reader. The resulting values were compared with a positive control (0.1 and 0.5 µM clotrimazole) and a negative control (no compound). IC_{50} values were determined according to the inhibition of metabolic activity or cell growth in the presence of a compound as compared to the non-inhibited negative control.

Results

Taxonomy of Producing Organism

Strain KF501 was isolated from water samples taken in the German Wadden Sea. The strain grew well in casamino acids glucose, Czapek and malt extract medium, slowly in potato-carrot medium and did not grow in liquid modified Wickerham medium. In general, the growth of strain KF501 was relatively slow by comparison with other fungal isolates.

Macroscopic and microscopic morphological features, colony color and structure of conidiophores were characteristic of *Cladosporium* spp. (Samson et al. 2000). This classification was corroborated by the comparison of the ITS DNA sequence of strain KF501 with sequences available at GenBank employing the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). The search resulted in 100% similarity to *Cladosporium cladosporioides*, *C. pseudocladosporioides*, *C. uredinicola*, *C. bruhnei* and *C. colombiae*. Hence, strain KF501 could be identified as a member of the genus *Cladosporium*, but identification to the species level was not possible.

Structure Elucidation

The new malettinin E (**4**) along with the known malettinins A–C (**1**–**3**) was obtained from the mycelia extracts of *Cladosporium* sp. strain KF501 grown in casamino acids glucose medium. The compounds were isolated by preparative reversed-phase HPLC. The structures of compounds **1**–**3** were readily identified by spectroscopic methods including ^{1}H NMR, UV and MS spectra (Angawi et al. 2003, Angawi et al. 2005).

Table III.2. Physico-chemical properties of malettinin E (**4**).

	Malettinin E (4)
Appearance	Orange crystals or amorphous solid (MeOH)
Molecular formula	$C_{16}H_{20}O_5$
Molecular weight	292
HRESIMS (*m*/*z*)	
Measured $[M + H]^+$	293.1391
Calculated $[M + H]^+$	293.1384
UV λ_{max} (MeOH) nm (log ε)	253 (4.45), 328 (3.68), 359 (3.91)
Mp (°C)	181–183 °C
$[\alpha]^{22}_D$	+58 (*c* 0.2, MeOH)

Malettinin E (**4**) was assigned a molecular formula of $C_{16}H_{20}O_5$ based upon HRESIMS. The physico-chemical properties of **4** are summarized in Table III.2. The molecular formula of **4** being the same as for **2** and **3** suggested the compounds to be isomeric. In addition, the one- and two-dimensional NMR spectra of **4** (^{1}H, ^{13}C, DEPT, COSY, HSQC and HMBC; see Table III.3 and Supporting Information) revealed the presence of considerable structural similarities with **2** and **3** (Table III.4) (Angawi et al. 2005). The ^{1}H and ^{13}C NMR data of **4** showed resonances for three methyl, two methylene and two aliphatic methine groups, one oxygenated methine and one carbonyl group as well as signals for six aromatic carbons and one oxygenated quaternary carbon. Thus, all structural groups and atoms present in **2** or **3** were also observed for **4**. Correlations of the aromatic protons in the HMBC NMR spectrum (Figure III.2) established the same tropolone moiety for **4** as found in the known malettinins. In addition to the tropolone unit, **4** contained a dihydropyran ring identical to that of **2** and **3**. The proton and carbon chemical shifts of the corresponding signals were in good agreement with those reported for **2** and **3**, with the proton signals agreeing particularly well to the structure

of **3**. HMBC couplings from H_2-7 to C-5, C-6 and C-15 gave evidence for the attachment of the dihydropyran ring to the tropolone moiety. The NMR data determined the remaining structural part of **4** to be a tetrahydrofuran ring connected to the dihydropyran unit via C-9 (Figure III.2). As a result, compound **4** was proven to have the same planar structure as described for **2** and **3**. The vicinal $J_{H7–H8}$ values of **4** (5.7 and 10.3) showed better correlations with those of **3** (5.9 and 13) than with those of **2** (6.2 and 3.8), suggesting a configuration like in **3** for the stereogenic center at C-9. However, the structures were not identical as considerable deviations in the chemical shifts of the proton and carbon signals of CH-12 (δ_H 2.23, δ_C 43.0) and CH-13 (δ_H 3.96, δ_C 86.0) in **4** were observed when compared to the reported shifts of the respective signals in **3** (δ_H 2.5, δ_C 34.7 and δ_H 4.32, δ_C 73.0, respectively) (Angawi et al. 2005). Taken the NMR data together, it seemed most likely that **4** was a stereoisomer of malettinin C (**3**), differing in the configuration at C-12 and/or C-13.

Table III.3. ^{1}H and ^{13}C NMR spectroscopic data of malettinin E (**4**) (600 MHz and 150 MHz) in methanol-d_4.

Position	**δ_C, Type**	**δ_H, Mult. (*J* in Hz)**	**COSY**	**HMBC**
1	115.4, CH	6.92, s		2, 3, 6, 7, 15
2	166.7, C			
3	173.0, C			
4	125.7, CH	7.13, s	16	2, 3, 5, 6, 15, 16
5	152.0, C			
6	124.0, C			
7a	33.2, CH_2	2.78, dd (17.5, 5.7)	7b, 8	5, 6, 8, 9, 15, 17
7b		2.51, dd (17.5, 10.3)	7a, 8	5, 6, 8, 9, 15, 17
8	31.0, CH	2.25, m[a]	7a, 7b, 17	6, 7, 9, 17
9	111.7, C			
11a	73.2, CH_2	4.08, t (8.1)	11b, 12	9, 12, 13, 18
11b		3.62, t (9.2)	11a, 12	12, 13, 18
12	43.0, CH	2.23, m[a]	11a, 11b, 13, 18	11, 13, 18
13	86.0, CH	3.96, d (7.8)	12	8, 9, 11, 12, 18
15	161.8, C			
16	27.2, CH_3	2.41, s br	4	4, 5, 6, 7, 15
17	15.8, CH_3	1.143, d (6.5)	8	6, 7, 8, 9
18	14.6, CH_3	1.145, d (6.8)	12	11, 12, 13

[a]Signal deduced from the HSQC NMR spectrum.

Figure III.2. Selected HMBC (arrows) and COSY (bold) correlations for **4**.

Table III.4. ^{13}C and ^{1}H NMR spectroscopic data of malettinin B (**2**) and C (**3**) according to Angawi et al. (2005) and malettinin E (**4**).

	Malettinin B (2)		**Malettinin C (3)**		**Malettinin E (4)**	
Position	**δ_C[a]**	**δ_H, Mult. (*J* in Hz)[b]**	**δ_C[c]**	**δ_H, Mult. (*J* in Hz)[b]**	**δ_C[d]**	**δ_H, Mult. (*J* in Hz)[d]**
1	112.6	6.76, s	113.2	6.95, s	115.4	6.92, s
2	164.1		164.4		166.7	
3	173.0		171.7		173.0	
4	125.0	7.01, s	125.0	7.06, s	125.7	7.13, s
5	151.2		149.8		152.0	
6	120.2		122.2		124.0	
7a	33.9	3.02, dd (17, 6.2)	32.0	2.80, dd (17, 5.9)	33.2	2.78, dd (17.5, 5.7)
7b		2.56, dd (17, 3.8)		2.52, dd (17, 13)		2.51, dd (17.5, 10.3)
8	28.8	2.57, m	29.9	2.22, m	31.0	2.25, m
9	113.1		106.2		111.7	
11a	74.5	4.18, t (7.8)	73.7	4.12, dd (8.8, 7.7)	73.2	4.08, t (8.1)
11b		3.64, dd (10, 7.8)		3.65, dd (8.8, 5.8)		3.62, t (9.2)
12	36.8	2.70, m	34.7	2.50, m	43.0	2.23, m
13	76.6	3.90, d (4.8)	73.0	4.32, d (8.7)	86.0	3.96, d (7.8)
15	160.3		158.3		161.8	
16	27.2	2.36, s	26.9	2.40, s	27.2	2.41, s br
17	14.3	1.01, d (6.6)	15.0	1.08, d (6.6)	15.8	1.143, d (6.5)
18	10.3	1.04, d (6.6)	12.8	1.21, d (7.2)	14.6	1.145, d (6.8)

[a]Recorded in acetone-d_6 at 90-100 MHz. [b]Recorded in acetone-d_6 at 400 MHz. [c]Recorded in chloroform-d_1 at 90-100 MHz. [d]Recorded in methanol-d_4 at 600 MHz or 150 MHz, respectively.

In order to determine the relative configuration of **4** conclusively, a single-crystal X-ray diffraction analysis was performed. It showed that the compound consisted of two crystallographically distinct molecules (**4a** and **4b**) with identical relative configuration exhibiting slight conformational differences between the molecules (Figure III.3). The X-ray crystal structure of **4** confirmed the assumptions made on the basis of the NMR data as it was found to be identical to **3** except for the configuration at C-13. Thus, **4** was identified to be the C-13 epimer of **3** as shown in Figure III.1.

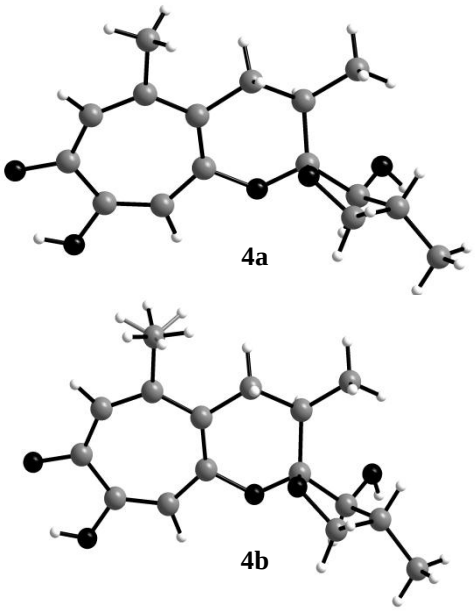

Figure III.3. Crystal structures of the two crystallographically distinct molecules of malettinin E (**4a** and **4b**). The H atoms of one of the methyl groups in **4b** are disordered.

Biological Activities

Compounds **1**–**4** were tested for biological activities against a set of bacterial and fungal test strains. The malettinins were found to be bioactive against the plant pathogenic bacterium *Xanthomonas campestris* and the human pathogenic dermatophyte *Trichophyton rubrum* (Table III.5). Compounds **2**–**4** showed inhibition of *X. campestris*, whereas **1** did not inhibit the bacterial test strain notably. The IC_{50} values of **2**–**4** were in approximately the same concentration range (28.3-37.9 µM). With regard to *T. rubrum*, all tested malettinins **1**–**4** exhibited inhibitory activities. However, the strength of inhibition differed between the compounds, with the new malettinin (**4**) and **1** showing highest activities (IC_{50} of 30.7 and 33.1 µM), while **2** was half as active (IC_{50} of 60.6 µM) and **3** was even less active (IC_{50} of 83.2 µM).

Beside the reported antifungal and antibacterial properties in Table III.5, malettinins B and C (**2** and **3**) showed weak inhibition (<80%) of *Staphylococcus epidermidis*, *Bacillus subtilis* and *Candida albicans* at a test concentration of 100 µM. At the same concentration, malettinin E (**4**) exhibited weak inhibitory effects on the metabolic activity of *S. epidermidis* and *C. albicans*. Malettinin A did not show antibacterial effects, but also inhibited *C. albicans* (81% inhibition at 100 µM). As these activities were poor, IC_{50} values were not determined.

Table III.5. IC_{50} values for antibacterial and antifungal activities of malettinins A–C and E (**1**–**4**).

Compound	*X. campestris* IC_{50} [µM]	*T. rubrum* IC_{50} [µM]
Malettinin A (**1**)	> 100	33.1 (±4.6)
Malettinin B (**2**)	28.3 (±5.4)	60.6 (±2.3)
Malettinin C (**3**)	37.9 (±3.8)	83.2 (±3.0)
Malettinin E (**4**)	28.7 (±1.7)	30.7 (±0.2)
Chloramphenicol	2.1 (± 0.6)	ND
Clotrimazole	ND	0.2 (± 0.0)

Abbreviation: ND, not determined.

Discussion

For the first time, malettinins A–C and E (**1**–**4**) were isolated from a fungus belonging to the genus *Cladosporium*. Originally, **1**–**3** were purified from an unidentified fungal colonist, which additionally produced a forth related metabolite called malettinin D (Angawi et al. 2003, Angawi et al. 2005). Malettinin D has not been detected in the culture extracts of *Cladosporium* sp. strain KF501, instead a new 13-epimer of **3**, named malettinin E (**4**), was found to be produced. **1**–**4** are members of the family of tropolone fungal metabolites containing an α-tropolone substructure. Even though many derivatives possessing a partial structure of this tropolone type are described for plants, only few such compounds have been reported within the fungal kingdom. Representative structures of fungi are puberulic acid, puberulonic acid, stipitatic acid and related structures (Corbett et al. 1950, Iwatsuki et al. 2011). More complex known compounds are epolone A or B, pycnidione, eupenifeldin or fusariocin C (Ito et al. 1981, Mayerl et al. 1993, Cai et al. 1998), which structurally share the fused tropolone/dihydropyran ring structure with the malettinins. However, malettinins **1**–**4** are unique with regard to their linkage of the tropolone/dihydropyran ring to a furan.

Malettinins A–C (**1**–**3**) were described as displaying weak inhibitory properties against *C. albicans, B. subtilis* and *S. aureus* employing disc diffusion assays (Angawi et al. 2003, Angawi et al. 2005). In addition, **1** inhibited *Aspergillus flavus* and *Fusarium verticillioides* (Angawi et al. 2003). Even though the activities against *C. albicans*, *B. subtilis* and antistaphylococcal activities against *S. epidermidis* were observed for **2** and

3 in this study as well, higher activities were found when profiled against the bacterium *X. campestris* and the fungus *T. rubrum*. *X. campestris* causes a variety of plant diseases, e.g., black rot in cabbage which is difficult to treat and therefore leads to high crop losses (Roohie and Umesha 2012). *T. rubrum* is responsible for clinical pictures like tinea pedis (athlete's foot) and likewise treatments are challenging, in part due to the toxicity in long-term treatments (Ramsey et al. 2013). New bioactive compounds for better treatments in plant protection as well as in clinical aspects are hence highly desirable. Comparing activities though shows that the malettinins inhibit *X. campestris* with a 13-fold higher IC_{50} than the antibiotic chloramphenicol used as positive control. Thus, their activities can be considered as moderate, only. The inhibition of the malettinins against *T. rubrum* is rather poor given that the IC_{50} of the positive control clotrimazole is lower by a factor of greater than 170.

The activities regarding *X. campestris* showed that all stereoisomeric compounds (**2**–**4**) inhibited the test strain in comparable concentration ranges, while **1**, possessing a very slightly varied furan ring, did not show inhibition. This observation suggests that the furan ring of the malettinins is critical for antibacterial properties against *X. campestris*. With regard to activities against *T. rubrum*, apparently configurational changes of the malettinins determine the strength of activity, since the distinct stereoisomers exhibited different IC_{50} values.

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GENERAL DISCUSSION

Natural products isolated from fungi have proved very valuable for mankind, owing to their use, e.g., in medicine and agriculture. However, the low-hanging fruits in natural product discovery have been picked and it becomes an increasingly difficult undertaking to find new bioactive compounds. Lower hit rates and the re-discovery of known compounds, amongst others, drive up the costs for the discovery and development of drugs derived from natural products. Pharmaceutical companies have thus changed their strategic orientation und turned their focus to combinatorial chemistry. With the synthesis of new organic compounds by the combination of a set of smaller chemical structures, libraries comprising millions of compounds can be generated within a short time and are screened for bioactivities in high-throughput screenings. Yet the success of this method has not materialized to date as only one *de novo* drug (sorafenib) has emerged from synthetic-combinatorial libraries in the last 30 years (Newman and Cragg 2012). Thus, in comparison, 0.001% of compounds synthesized by combinatorial chemistry became accepted drugs, while of the known microbial metabolites about 0.2–0.3% became a therapeutic product and about the same number served as lead compounds for the development of new drugs. Therefore, microbial natural products show a more than two order of magnitude higher efficacy than compounds originating from combinatorial chemistry (Bérdy 2005). These figures paired with the increasing resistances toward current drugs and newly emerging pathogens demand concerted efforts in the drug discovery from natural products.

The aim of the present PhD thesis was thus to find new fungal natural products by creating a screening strategy that allows picking the “high-hanging fruits”. For this purpose, different approaches approved for natural product discovery were combined (fungal isolates from little-explored ecosystems, less studied genera, genetic potential according to PKS and NRPS gene sequences and stimulating effects on biosynthesis by variation of culture conditions) and used for a rational selection of fungi which were in detail investigated with regard to their biosynthesized natural products. In this way, three new cyclodepsipeptides, five new linear and macrocyclic polyesters and one new tropolone structure were identified and characterized. Given that as a general rule up to 10,000 microbial extracts need to be analyzed to discover one to ten new bioactive structures (Gräfe 1999), the respective ratio in the studies presented here is impressively high. From only 243 extracts, nine new natural products including five active

compounds were described, proving the screening strategy employed very effective. In addition, even if taking into account that some of the nine compounds are structurally related, the number of three different structural classes isolated by a screening of 243 extracts still presents a fairly good ratio.

Unlike other organisms, microbes colonize all living and non-living niches on Earth such as arctic, antarctic and alpine regions, deserts, deep rock sediments and marine environments including the Wadden Sea (Gunatilaka 2006). According to literature, it is for the first time that fungal natural products isolated from Wadden Sea waters were described. However, such a literature survey might not totally depict the situation as examples are known where the isolation site was not specified to the Wadden Sea, but the North Sea in general (Schulz et al. 2011). As had been demonstrated for bacteria before (Liang 2003, Bitzer et al. 2006, Grossart et al. 2009), it was shown that fungal strains originating from the Wadden Sea possess the potential to produce a diverse range of natural products. Notably, the fungi were isolated from water samples, which usually are not a frequent source for strains reported to produce new natural products (Figure 4). Approximately two-thirds of all new compounds produced by marine-derived fungi originate from strains isolated from plant and animal sources. Concerning the non-living sources, sediments form the largest segment, while fungi derived from water samples only account for 1% of newly characterized natural products (Rateb and Ebel 2011).

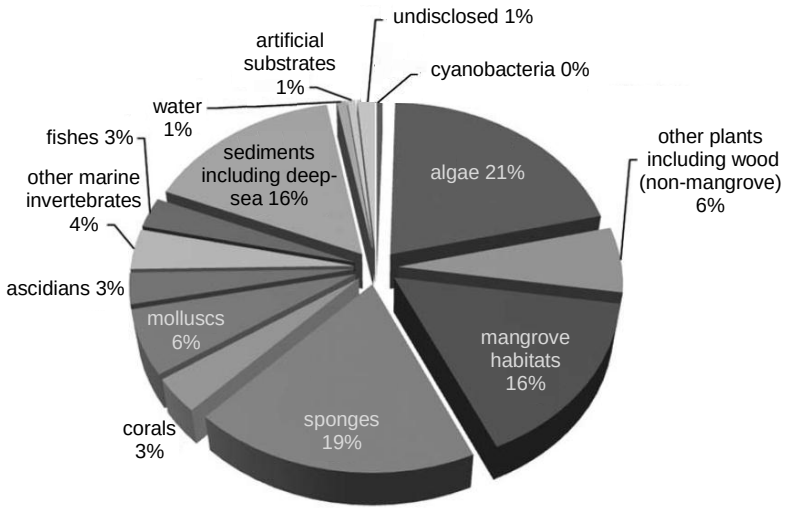

Figure 4. New compounds from marine-derived fungi until mid-2010, divided by sources of the producing strains (according to Rateb and Ebel 2011).

Though isolated from the Wadden Sea, it remains unclear whether the fungal isolates investigated in the studies were obligate or facultative marine organisms or if they were isolated as dormant spores from the water. The Wadden Sea as a land/ocean interface, of course, receives input of terrestrial and freshwater fungi, for instance, by river inflow or via air (especially important for fungal spores). According to Kohlmeyer and Kohlmeyer (1979), obligate marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat, whereas facultative marine fungi are those from freshwater or terrestrial areas able to grow (and possibly also to sporulate) in the marine environment. The fungal isolates described in chapters I–III could be facultative marine organisms as they grew on modified casamino acids glucose medium not containing sodium chloride as well as on modified Wickerham medium with sodium chloride supplemented. Nevertheless, *Calcarisporium* and *Cladosporium* strains belong to ubiquitous genera common to terrestrial habitats and no definite statement can be made unless actual growth in sea water is tested (Shearer et al. 2007).

Furthermore, the results of the study show that it is worth to have a look for novel natural products in less studied fungal genera. However, with only two strains, namely a *Calcarisporium* sp. and a *Cladosporium* sp., it is a small number of organisms that produced new natural products. In this regard, the fact that the initial collection of fungal isolates lacked high diversity is considered an important reason. Even though the total number of strains screened was large (109 strains), 30 isolates belonged to the particularly well-investigated genus *Penicillium* and another 30 isolates were yeasts which rarely produce bioactive compounds (Bérdy 2005). The search for new natural products in not extensively investigated genera is a screening approach that is presumably more promising when applied to a more diverse group of fungal isolates. Therefore, the isolation procedure is of crucial importance, requiring the employment of various different isolation techniques. In addition, the selection of fungi from biodiversity "hotspots" such as mangrove habitats (Shearer et al. 2007) or from sponges, which are supposed to host unique and diverse fungal communities (Bugni and Ireland 2004, Wang 2006, Wiese et al. 2011), as an additional criterion for the screening would probably enhance the screening output.

While microbial natural product discovery for decades was a rather coincidence-driven culturing and screening of thousands of strains, the advances in genome sequencing offer more directed possibilities. Schneemann (2011) showed for actinomycetes that

strains selected based on the presence of biosynthetic genes for natural products according to molecular biological analysis produced higher numbers and more diverse natural products as compared to strains where no such biosynthetic genes were detectable. Similarly, a genetic screening for PKS and NRPS genes in the fungal isolates of this study was performed and proved a fast and useful tool to prioritize strains with the genetic capabilities to produce natural products. The increasing number of fungal genome sequencing data indicates that when a strain harbors one gene cluster for the biosynthesis of natural products, it is likely that further gene clusters are also present (Keller et al. 2005, Khaldi et al. 2010, Brakhage and Schroeckh 2011). Thus, a positive screening for PKS or NRPS genes can help to find interesting producing organisms. Nonetheless, it has also become clear that identifying the biosynthetic genes and pathways is much easier than finding the associated natural products (Jensen et al. 2013). Genomics-based investigation of natural product biosynthesis revealed that an even greater number of gene clusters stay silent in the laboratory than formerly thought (Hertweck 2009). This means that despite having access to microbial genomes nowadays, the induction of expression of the numerously proven gene clusters still represents one of the major bottlenecks in the research field and the process of natural product discovery therefore largely remains a matter of serendipity (Jensen et al. 2013). Various approaches can be followed to activate the silent gene clusters. Here, their expression was stimulated by the variation of the culture media as becomes obvious by the fact that all new compounds were isolated from cultures in casamino acids glucose medium, but not from the initial standard cultures (modified Wickerham medium). The approach offers infinite possibilities for continuation, e.g., the creation of culture media that mimic the natural parameters of the original habitat or the employment of epigenetic modifiers. Equivalently, alternative strategies for the activation of biosynthetic pathways, in particular by means of molecular genetic manipulation, exist. Especially for *Calcarisporium* sp. strain KF525 further investigations should be taken into consideration, because the strain produces compounds of different structural classes and may hence well have the potential to synthesize an even greater variety of natural products. First steps toward this direction have been undertaken as the genome of KF525 was sequenced and is currently evaluated with regard to biosynthetic gene clusters for natural products (unpublished data).

In addition, the genome sequence data of KF525 form the basis for future analysis on the biosynthetic mechanisms involved in the calcaripeptide production. According to

the biosynthetic studies performed, the calcaripeptides presumably belong to the structural class of PKS−NRPS hybrids. PKS−NRPS hybrid molecules were discovered in fungi only a decade ago and the programming of hybrid synthetases is much less understood than in bacteria. To date, approximately a dozen PKS−NRPS megasynthetases can be linked to the corresponding natural products (Boettger and Hertweck 2013). Typically, these hybrid enzymes consist of a type I iterative PKS module, which is followed by a single NRPS module fusing the polyketide chain to one amino acid (Fisch 2013). Two possible release mechanisms are currently discussed involving either a Dieckmann cyclization or a reductive release to form a free aldehyde that can undergo a Knoevenagel condensation (Figure 5) (Boettger and Hertweck 2013). As a result, the primary products of PKS−NRPS hybrid synthetases are acyltetramic acids, which can further be altered by additional enzymes to yield the final hybrid compound (Molnár et al. 2010).

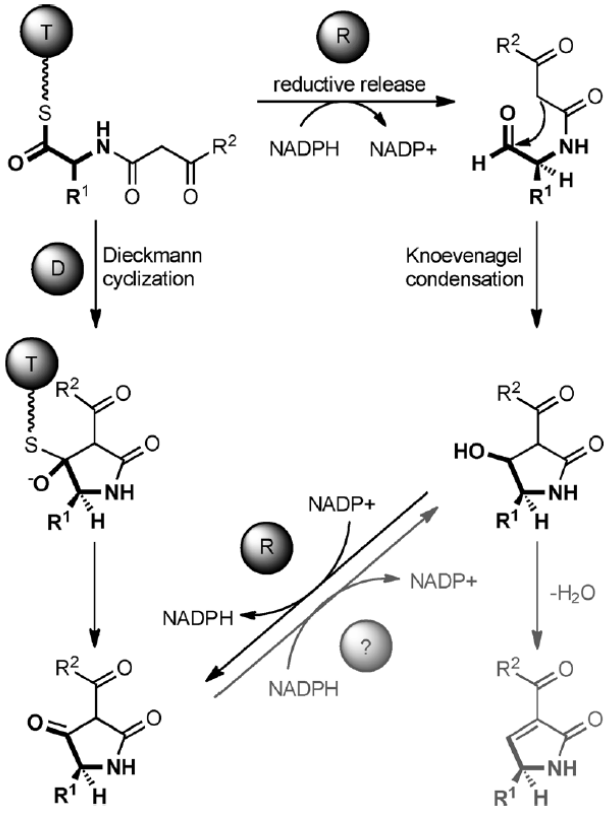

Figure 5. Possible release mechanisms of PKS–NRPS hybrids to yield tetramic acids (black) and pyrrolinones (gray) (according to Boettger and Hertweck 2013).

Remarkably, the calcaripeptides differ from this prototype of PKS−NRPS hybrids. First of all, they contain two amino acid moieties and second, showing no tetramic acid

structural nature, a Dieckmann cyclisation or Knoevenagel condensation during biosynthesis is unlikely. For the emericellamides which similarly contain more than one amino acid residue and are no tetramic acid derivatives, a biosynthetic route involving two distinct enzymes (one PKS and one NRPS) was described (Chiang et al. 2008). The exact release and cyclization mechanism in the formation of the emericellamides remains to be elucidated though, as the involved NRPS enzyme does not contain a TE-domain (Chiang et al. 2008). In fact, several other PKS−NRPS hybrids that are no derivatives of tetramic acid and contain one or more amino acids have been observed in fungi, but their biosynthesis is not understood (Ratnayake et al. 2008, Pittayakhajonwut et al. 2009, Vervoort et al. 2011, Guo et al. 2012, Krasnoff et al. 2012). By taking advantage of the genomic DNA data of strain KF525, the identification of the gene cluster encoding the calcaripeptide biosynthetic enzymes will hence not only bring light into their biosynthetic machinery, but more importantly also improve the general understanding of PKS−NRPS hybrid biosynthesis in fungi.

With respect to the application potential of the new compounds, malettinin E and the calcarides exhibited antibacterial and antifungal bioactivities against test strains relevant for human and plant diseases. Though moderately active in their natural forms, it remains to be evaluated if they have a potential as drugs and agrochemicals in a chemically modified form. Interesting bioactive properties of natural products are often not detected at first sight, as it is the case for the calcaripeptides. Despite their presumable role in specific biological interactions on the basis of bioactivities in the natural environment, only close to 50% of all known microbial metabolites display some kind of biological activity in laboratory experimental set-ups (Bérdy 2012). Given that the calcaripeptides have a high structural novelty, it could well be that the compounds possess unique bioactive properties. In order to find these, a testing of a broader range of targets is required. Found bioactive properties would then be all the more interesting as the so far performed assays support a specific activity profile for the calcaripeptides and many side effects, e.g., of cytotoxic nature, can most likely be ruled out already by now.

Certainly, the way from the first discovery of a new compound to an approved drug is long. The most advanced natural product of a marine-derived fungus in the drug pipeline is the diketopiperazine halimide discovered by Fenical et al. (1998) in the 1990s. The cytotoxic compound was isolated from an *Aspergillus* sp. collected in the

waters off the Philippine Islands. Halimide served as a lead structure for the closely related synthetic analog plinabulin which is currently undergoing phase II clinical trials in combination with docetaxel in patients with advanced non-small cell lung cancer (ClinicalTrials.gov 2013, Rateb and Ebel 2011). This example and the more than 1000 new natural products from marine-derived fungi point out the important role of this group of organisms for drug discovery (Rateb and Ebel 2011). Furthermore, the studies presented here clearly show that the potential for natural product biosynthesis in fungi from marine habitats is far from being exploited. To say it with Newman and Cragg (2012), “Much of Nature’s treasure trove of small molecules remains to be explored, particularly from the marine and microbial environments”.

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DECLARATION/ERKLÄRUNG

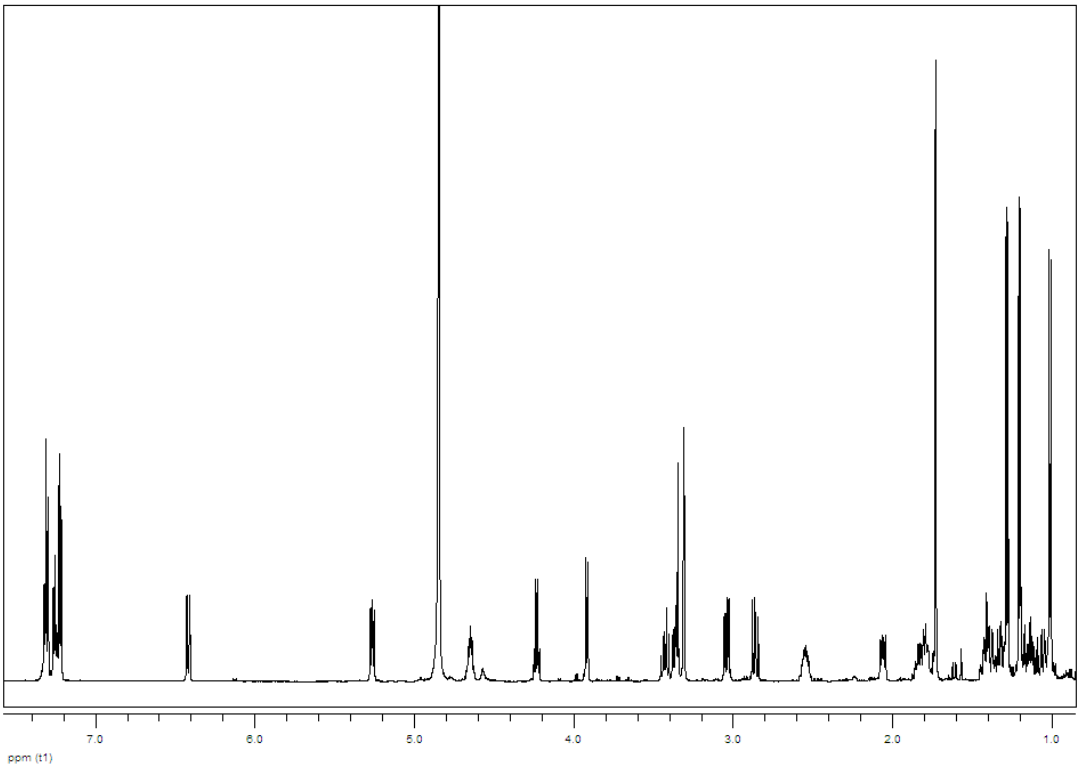
Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig und ohne unerlaubte Hilfe angefertigt habe und dass sie nach Form und Inhalt meine eigene Arbeit ist. Sie wurde weder im Ganzen noch zum Teil einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt und entstand unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft.

……..
(Johanna Silber)

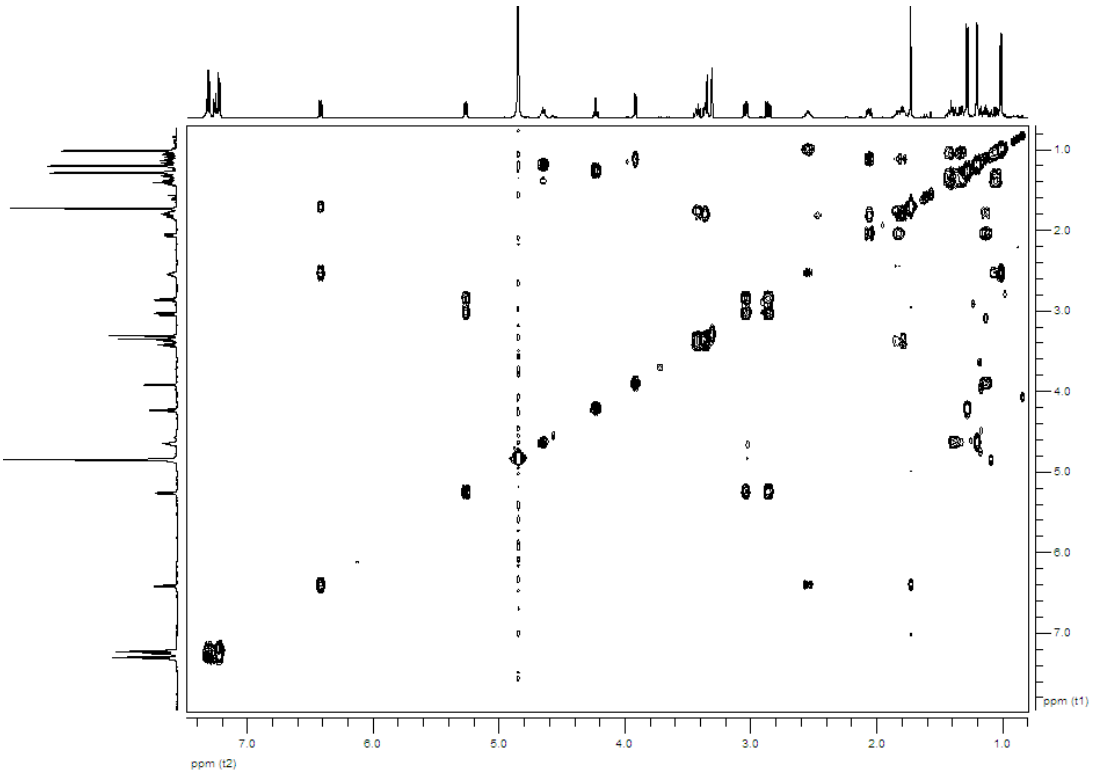
APPENDIX

Supporting Information for Chapter I

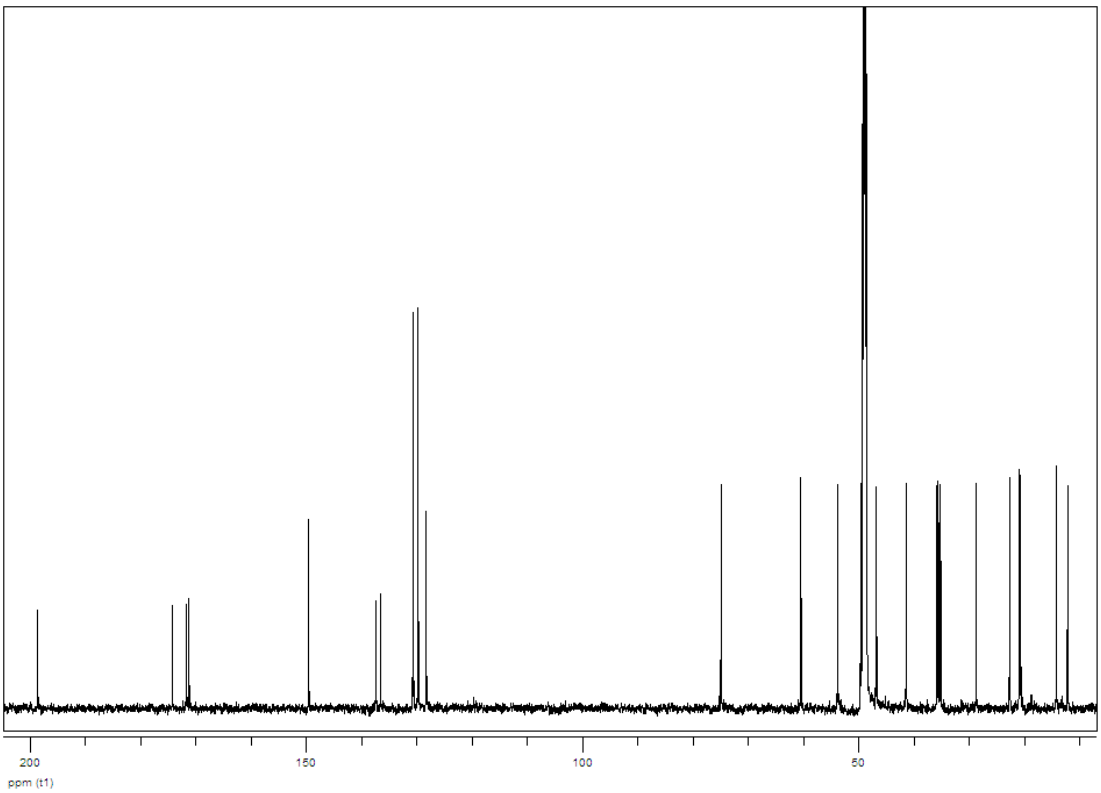
AI.1. ^{1}H NMR (600 MHz, methanol-d_4) spectrum of calcaripeptide A (**1**).



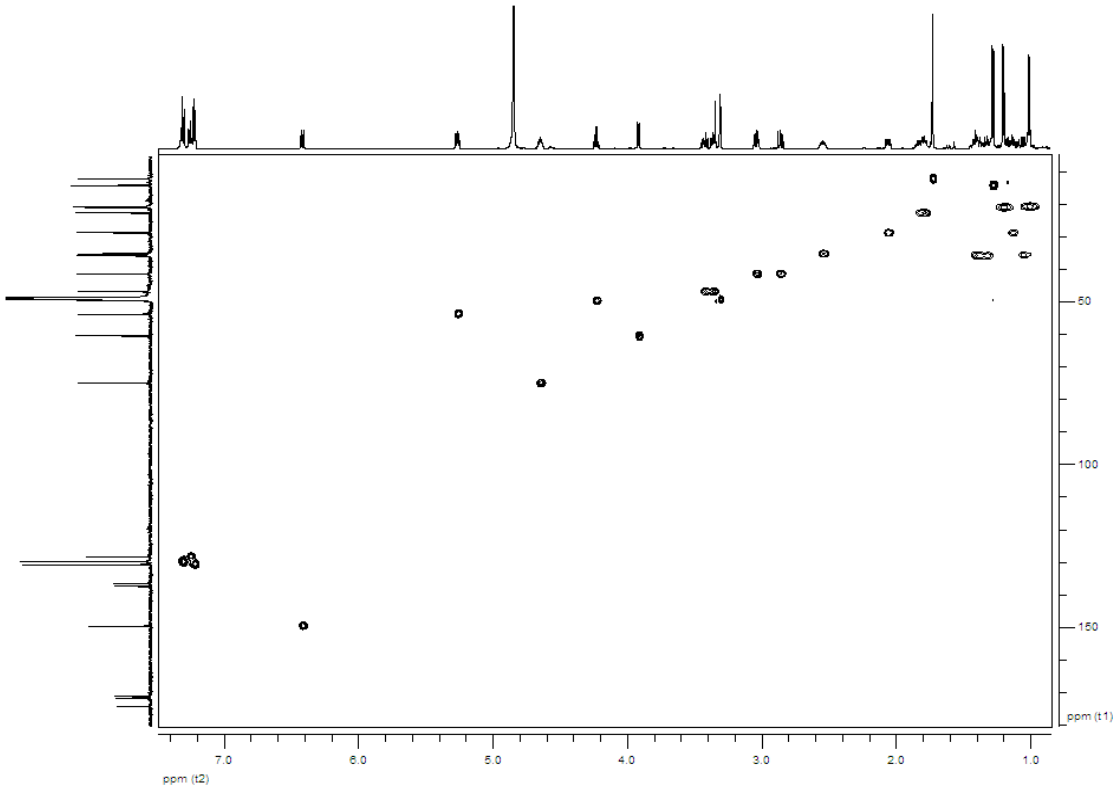
AI.2. COSY (600 MHz, methanol-d_4) spectrum of calcaripeptide A (**1**).



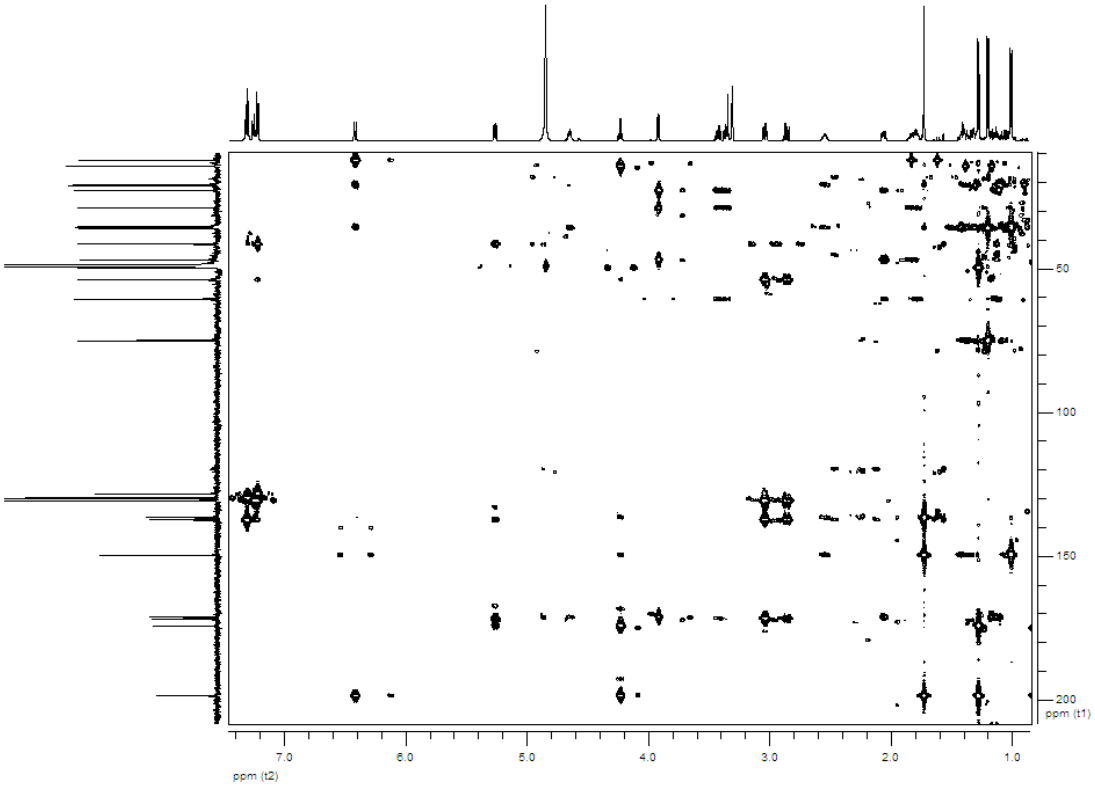
AI.3. ^{13}C NMR (150 MHz, methanol-d_4) spectrum of calcaripeptide A (**1**).



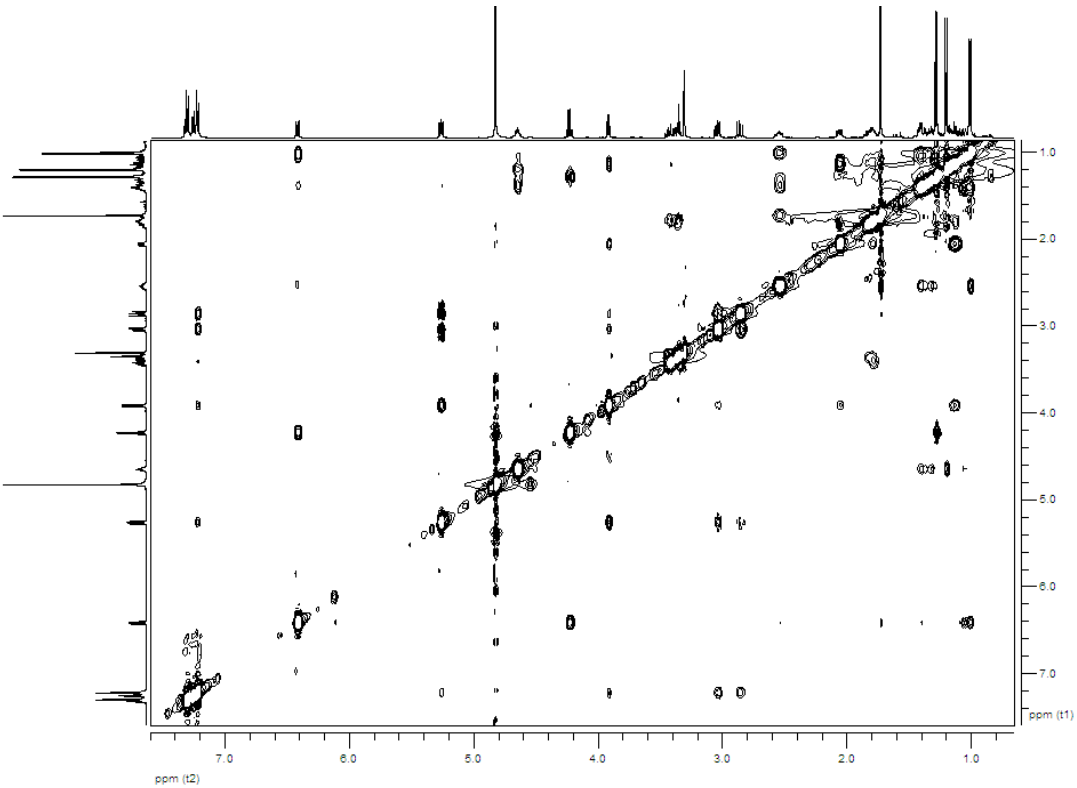
AI.4. HSQC (600 MHz, methanol-d_4) spectrum of calcaripeptide A (**1**).



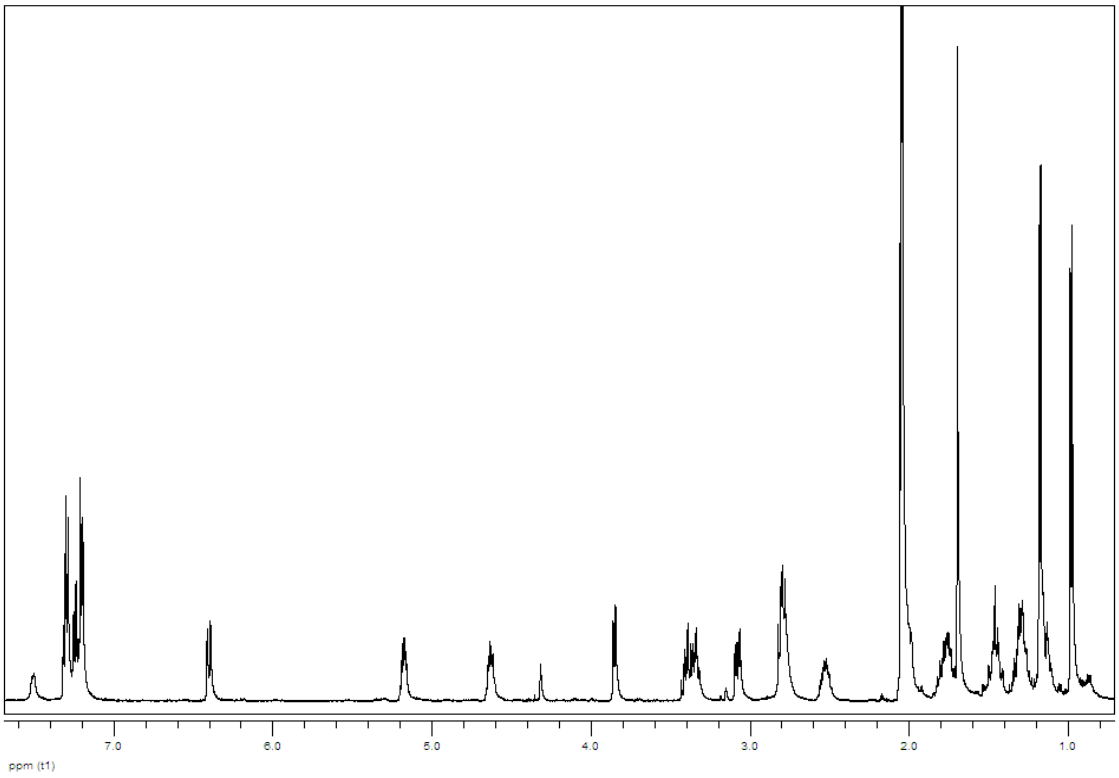
AI.5. HMBC (600 MHz, methanol-d_4) spectrum of calcaripeptide A (**1**).



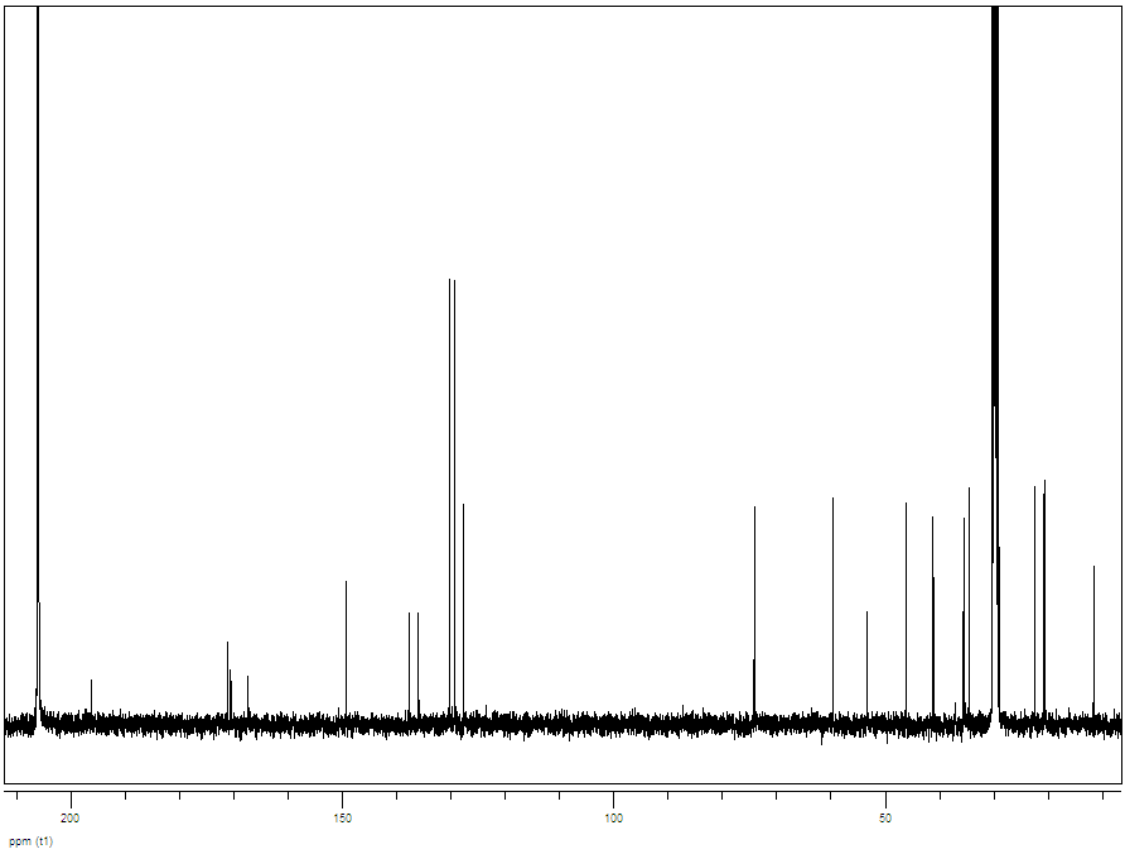
AI.6. NOESY (500 MHz, methanol-d_4) spectrum of calcaripeptide A (**1**).



AI.7. ^{1}H NMR (500 MHz, acetone-d_6) spectrum of calcaripeptide B (**2**).



AI.8. ^{13}C NMR (125 MHz, acetone-d_6) spectrum of calcaripeptide B (**2**).

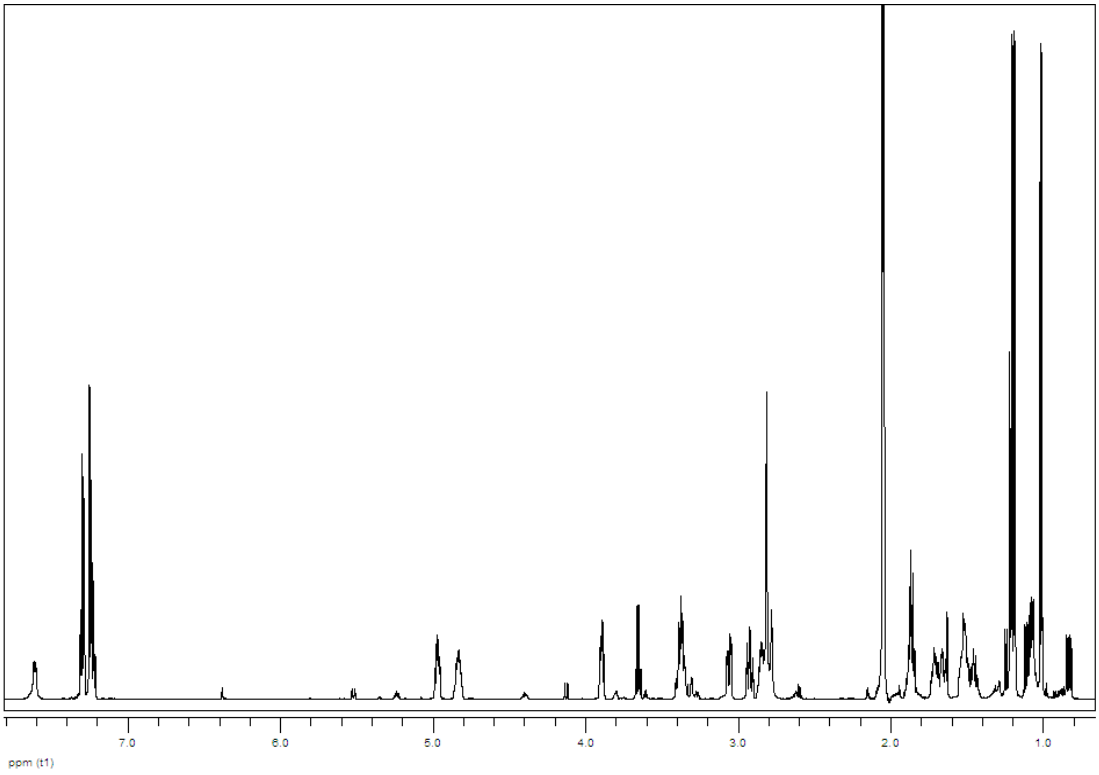


AI.9. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data of calcaripeptide B (**2**) in acetone-d_6.

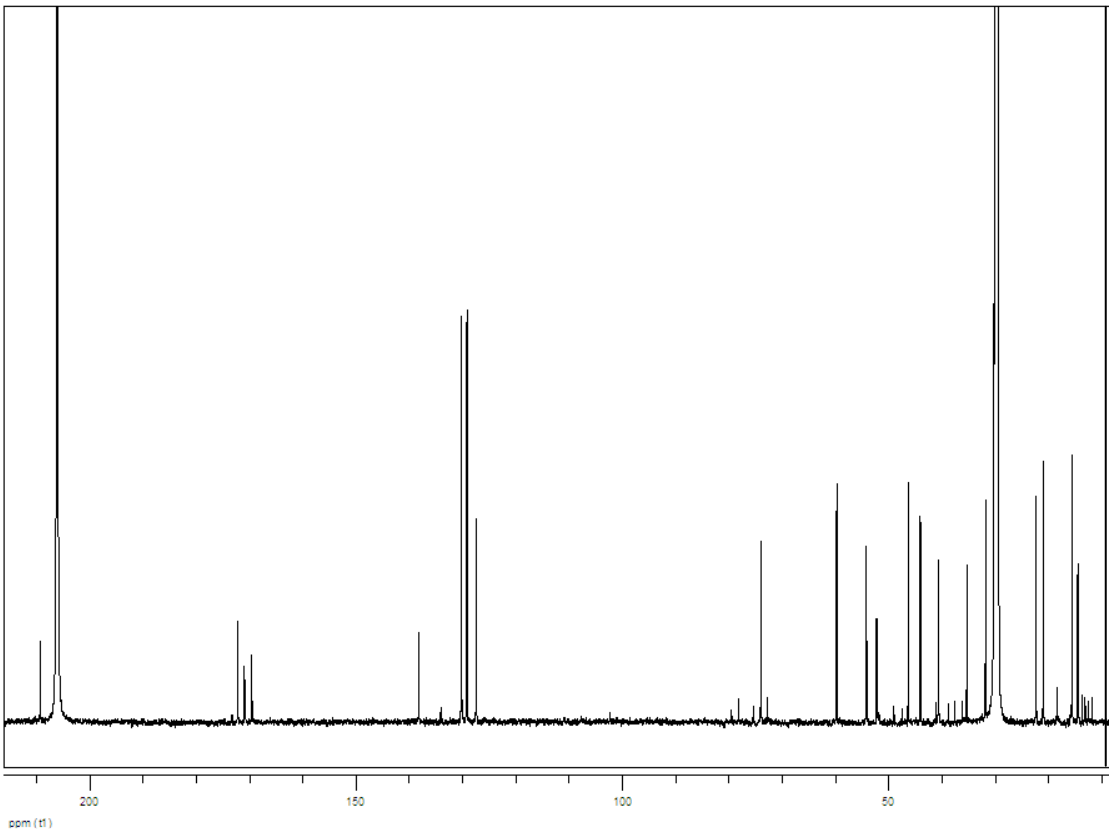
Position	δ_C, Type	δ_H, Mult. (*J* in Hz)	COSY	HMBC
1	167.5, C			
2a	48.2, CH_2[a]	4.32, br s	2b	1, 3
2b		3.15, br s	2a	1,3, 4
3	196.3, C			
4	136.0, C			
5	149.4, CH	6.40, br d (10.1)	6, 4-CH_3	3, 6, 7, 4-CH_3, 6-CH_3
6	34.6, CH	2.53, m	5, 7a, 7b, 6-CH_3	4, 7, 8, 6-CH_3
7a	35.4, CH_2	1.44, m[b]	6, 7b, 8a, 8b	5, 6, 8, 9, 6-CH_3
7b		1.15, m	6, 7a, 8a, 8b	5, 6, 8, 9
8a	35.6, CH_2	1.48, m[b]	7a, 7b, 8b, 9	6, 7, 9, 9-CH_3
8b		1.32, m	7a, 7b, 8a, 9	6, 7, 9-CH_3
9	74.1, CH	4.63, m	8a, 8b, 9-CH_3	8
4-CH_3	11.6, CH_3	1.70, d (1.3)	5	3, 4, 5, 6, 7, 6-CH_3
6-CH_3	20.6, CH_3	0.98, d (6.6)	6	5, 6, 7
9-CH_3	20.9, CH_3	1.18, d (6.2)	9	8, 9
1'	171.2, C			
2'	59.7, CH	3.85, br d (7.5)	3a', 3b'	1', 3', 4', 5'
3a'	29.0, CH_2	2.01[c]	2', 3b', 4'	1', 4', 5'
3b'		1.29, m	2', 3a', 4'	1', 2', 4'
4'	22.5, CH_2	1.77, m	3a', 3b', 5a', 5b'	2', 3', 5'
5a'	46.2, CH_2	3.39, m	4', 5b'	2', 3', 4', 1"
5b'		3.34, m	4', 5a'	2', 3', 4', 1"
1"	170.6, C			
2"	53.4, CH	5.18, m	3a", 3b", NH-2"	1, 1", 3", 4"
3a"	41.2, CH_2	3.08, dd (12.8, 5.0)	2", 3b", 5" + 9"	1", 2", 4", 5" + 9"
3b"		2.80, dd (12.8, 9.0)	2", 3a", 5" + 9"	1", 2", 4", 5" + 9"
4"	137.7, C			
5" + 9"	130.3, CH	7.20, br d (8.4)	3a", 3b", 6" + 8"	3", 5" + 9", 6" + 8", 7"
6" + 8"	129.3, CH	7.31, br dd (8.4, 7.4)	5" + 9", 7"	4", 5" + 9", 6" + 8", 7"
7"	127.7, CH	7.24, br t (7.4)	6" + 8"	4", 5" + 9"
NH-2"		7.51, br d (6.7)	2"	

[a]Signal deduced from the HSQC spectrum. [b]Proton signals of 7a and 8a overlap. [c]Signal partially obscured.

AI.10. ^{1}H NMR (600 MHz, acetone-d_6) spectrum of calcaripeptide C (**3**).



AI.11. ^{13}C NMR (150 MHz, acetone-d_6) spectrum of calcaripeptide C (**3**).



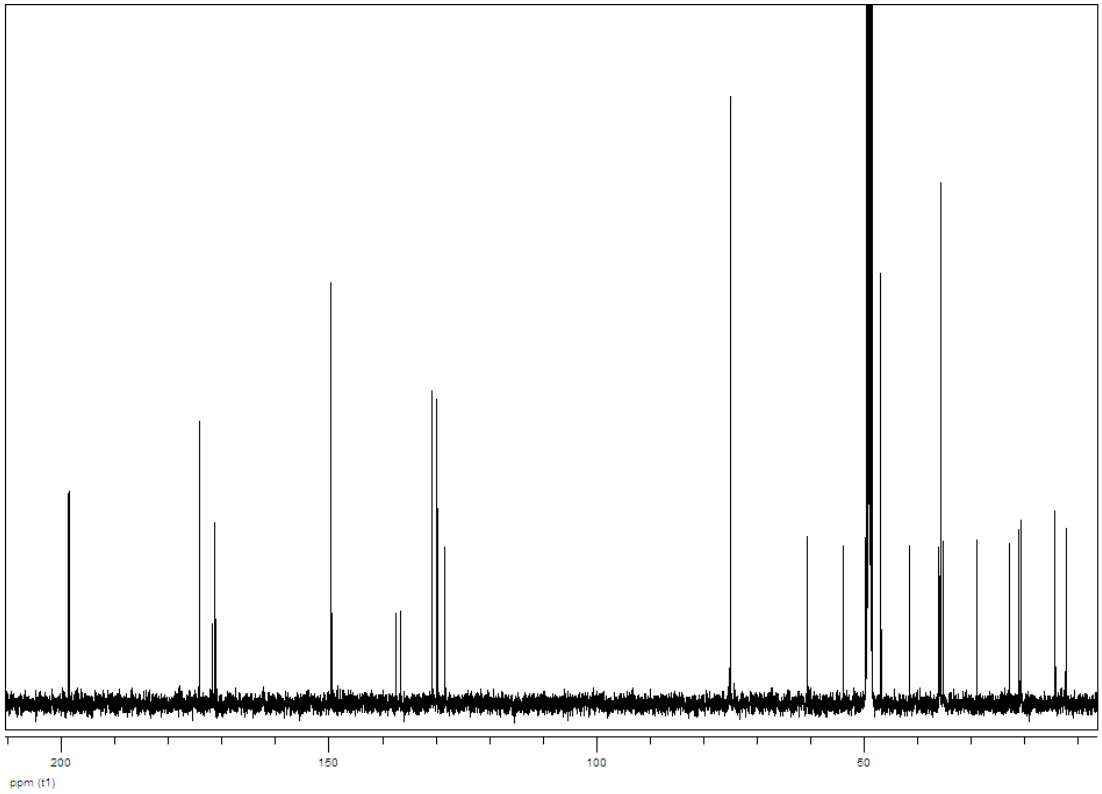
AI.12. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectroscopic data of calcaripeptide C (**3**) in acetone-d_6.

Position	**δ_C, Type**	**δ_H, Mult. (*J* in Hz)**	**COSY**	**HMBC**
1	171.0, C			
2	52.3, CH	3.66, q (6.9)	2-CH_3	1, 3, 4, 2-CH_3
3	209.3, C			
4	44.1, CH	2.85, m	5a, 5b, 4-CH_3	3, 5, 6, 4-CH_3
5a	31.8, CH_2	1.53, m[a]	4, 5b, 6a, 6b	3, 4, 6, 7, 4-CH_3
5b		1.06, m	4, 5a, 6a, 6b	3, 4, 6, 7, 4-CH_3
6a	35.3, CH_2	1.67, m	5a, 5b, 6b, 7	3, 4, 5, 7, 4-CH_3, 7-CH_3
6b		1.53, m[a]	5a, 5b, 6a, 7	3, 4, 5, 7, 4-CH_3, 7-CH_3
7	74.0, CH	4.84, m	5b, 6a, 6b, 7-CH_3	4, 5, 6, 4-CH_3, 7-CH_3, 1'
2-CH_3	14.5, CH_3	1.22, d (6.9)	2	1, 2, 3
4-CH_3	15.6, CH_3	1.02, d (6.7)	4	3, 4, 5
7-CH_3	21.0, CH_3	1.19, d (6.3)	7	6, 7
1'	172.2, C			
2'	59.8, CH	3.90, m	3a', 3b'	1', 3', 4', 5', 1"
3a'	30.3, CH_2	1.87, m	2', 3b', 4b', 5'	1', 2', 4', 5', 1"
3b'		1.46, m	2', 3a', 4b', 4a', 5'	1', 2', 4', 5'
4a'	22.4, CH_2	1.88, m	3b', 4b', 5'	1', 2', 3', 5'
4b'		1.71, m	3a', 3b', 4a', 5'	1', 2', 3', 5'
5'	46.3, CH_2	3.38, m	3a', 3b', 4a', 4b'	2', 3', 4', 1"
1"	169.6, C			
2"	54.2, CH	4.97, m	3a", 3b", NH-2"	1, 1", 3", 4"
3a"	40.6, CH_2	3.06, ddt (12.5, 4.7, 2.5)	2", 3b"	1", 2", 4", 5" + 9"
3b"		2.93, dddd (12.5, 10.0, 3.2, 2.1)	2", 3a"	1", 2", 4", 5" + 9"
4"	138.2, C			
5" + 9"	130.2, CH	7.25, br d (7.5)	6" + 8"	2", 3", 4", 5" + 9", 6" + 8", 7"
6" + 8"	129.2, CH	7.30, br t (7.5)	5" + 9", 7"	3", 4", 5" + 9", 6" + 8", 7"
7"	127.5, CH	7.23, br t (7.5)	6" + 8"	4", 5" + 9", 6" + 8"
NH-2"		7.61, br d (7.5)	2"	1, 1", 2"

[a]Proton signals of 5a and 6b overlap.

AI.13. Feeding experiment with 1-^{13}C-acetate:

^{13}C NMR (125 MHz, methanol-d_4) spectrum of calcaripeptide A (**1**).



Calculation of ^{13}C enrichment

^{13}C enrichment in calcaripeptide A (**1**) was calculated according to Scott et al. (1974). *J. Am. Chem. Soc.* 96(26), 8069-8080:

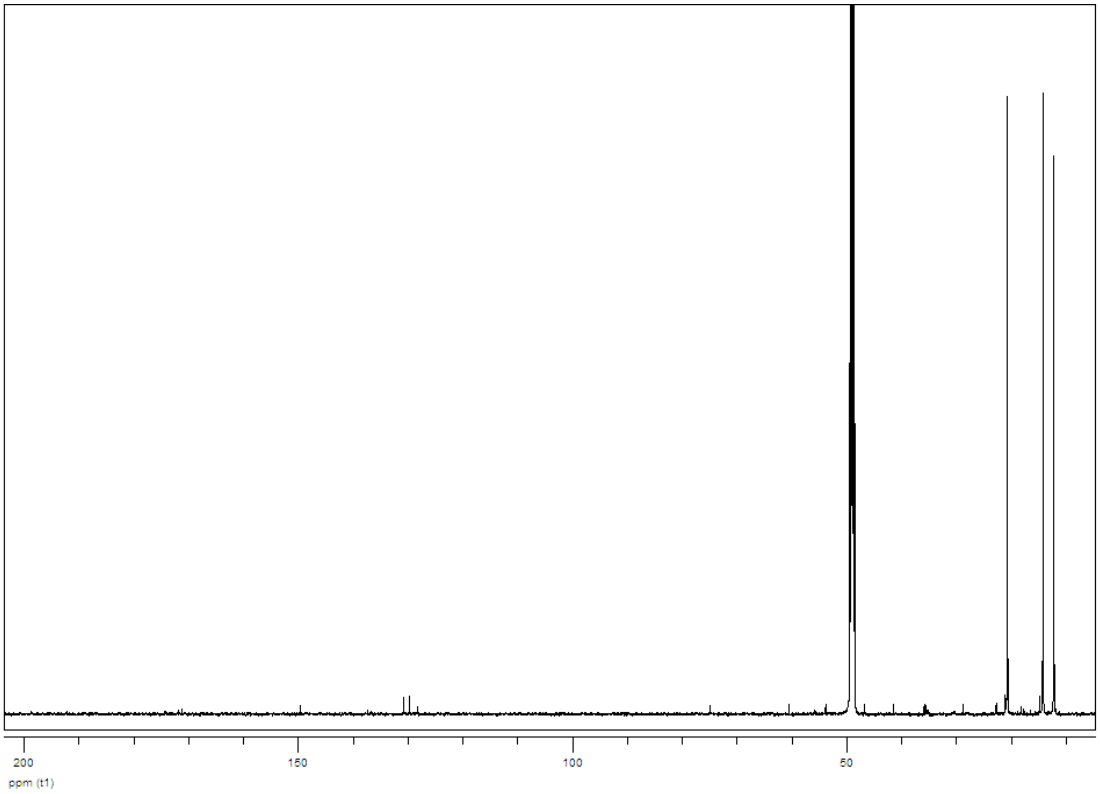
$$\text{Enrichment} = 1.1\% \times \frac{\text{integrated intensity } I \text{ [labeled]}}{\text{integrated intensity } I \text{ [unlabeled]}} - 1.1\%$$

Position	*I* [labeled]	*I* [unlabeled]	Enrichment [%]
1	1.92	0.45	3.6
3	1.80	0.45	3.3
5	4.00	0.89	3.8
7	4.41	1.17	3.0
9	4.07	1.03	3.2
1’	0.99	0.44	1.4
5’	2.68	1.14	1.5

For comparison of integrated intensities the integral of the signal of C-2’ was set to 1.00000 in the spectra of both labeled and unlabeled **1**.

AI.14. Feeding experiment with L-methionine-methyl-^{13}C:

^{13}C NMR (150 MHz, methanol-d_4) spectrum of calcaripeptide A (**1**).



Calculation of ^{13}C enrichment

^{13}C enrichment in calcaripeptide A (**1**) was calculated according to Scott et al. (1974). *J. Am. Chem. Soc.* 96(26), 8069-8080:

$$\text{Enrichment} = 1.1\% \times \frac{\text{integrated intensity } I \text{ [labeled]}}{\text{integrated intensity } I \text{ [unlabeled]}} - 1.1\%$$

Position	*I* [labeled]	*I* [unlabeled]	Enrichment [%]
2-CH_3	67.78	1.15	63.7
4-CH_3	57.60	1.00	62.3
6-CH_3	67.17	1.20	60.5

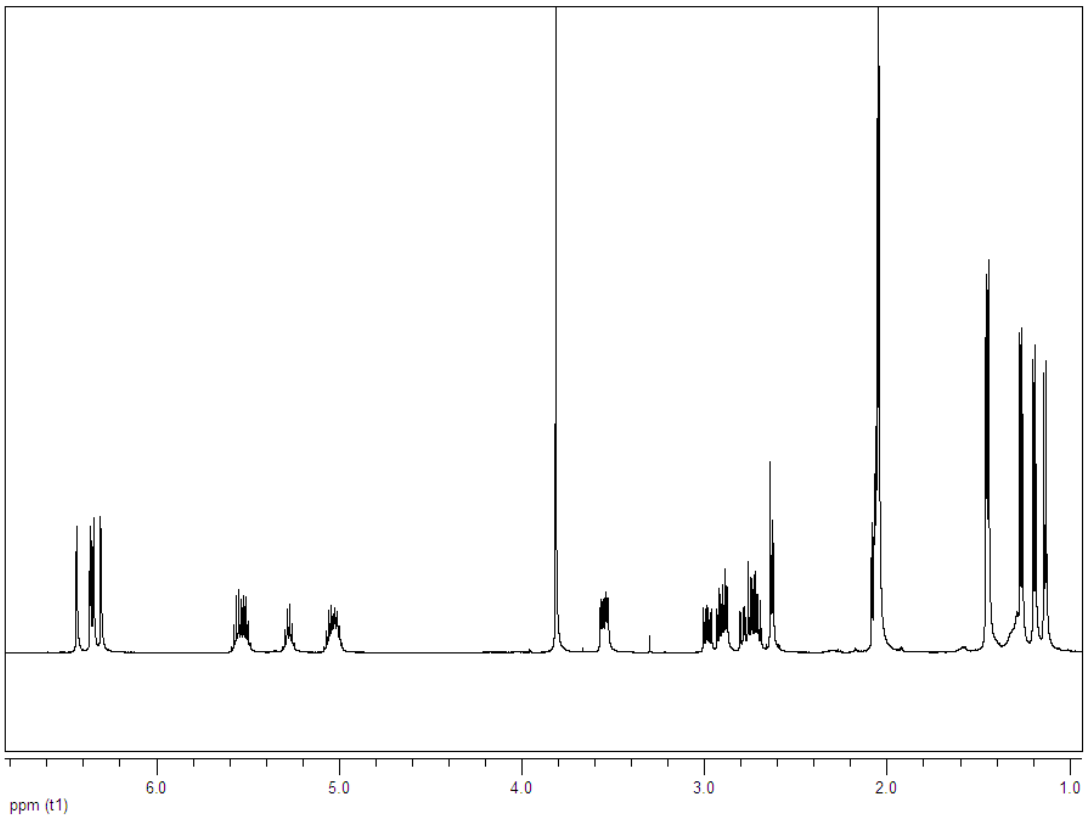
For comparison of integrated intensities the integral of the signal of C-2' was set to 1.00000 in the spectra of both labeled and unlabeled **1**.

AI.15. Activity assays - test strains, cell lines and enzymes.

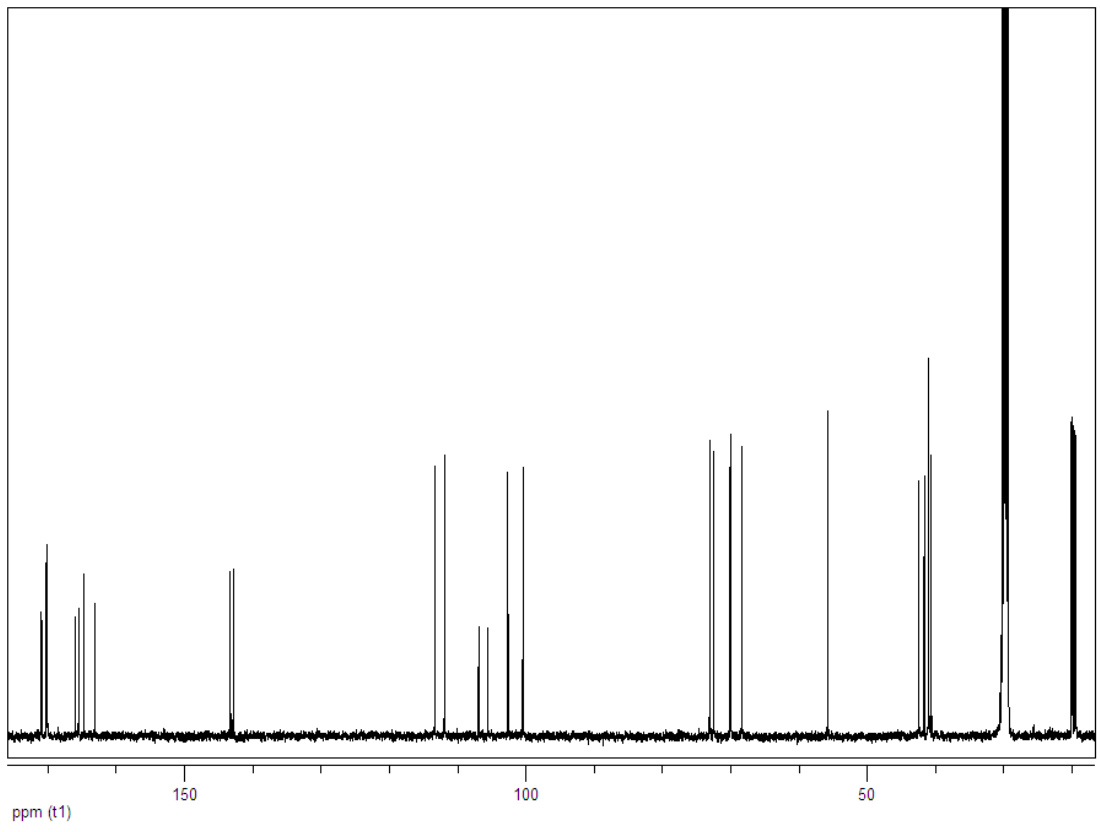
Calcaripeptides A–C (**1**-**3**) were tested for antibacterial, antifungal and cytotoxic properties and for inhibition of selected enzyme targets. The tested strains, cell lines and enzymes were as follows (compound concentration in assay): *Bacillus subtilis* DSM 347 (100 µM), *Staphylococcus lentus* DSM 6672 (100 µM), *Staphylococcus epidermidis* DSM 20044 (100 µM), *Xanthomonas campestris* DSM 2405 (100 µM), *Propionibacterium acnes* DSM 1897 (100 µM), *Candida albicans* DSM 1386 (100 µM), *Septoria tritici* (100 µM), *Phytophthora infestans* (50 µM), *Trichophyton rubrum* (100 µM), HepG2 cell line (50 µM), KIF5 fibroblasts (50 µM), glycogen synthase kinase-3*β* (10 µM), acetylcholinesterase (10 µM), phosphodiesterase 4B2 (10 µM) and protein tyrosine phosphatase 1B (10 µM). Moreover, calcaripeptide A (**1**) was tested negative for activities against *Ralstonia solanacearum* DSM 9544 (100 µM), in enzyme assays for reverse transcriptase (10 µM), histone deacetylase 6 (100 µM), histone deacetylase 8 (100 µM) and against the cell lines 786-0, MCF-7, M14, HL-60(TB), CCRF-CEM, K-562, MOLT-4, SR, A549/ATCC, COLO 205, HT29, KM12, SW-620, SF-268, SF-539, SK-MEL-5, OVCAR-3, OVCAR-8, NCI/ADR-RES, SK-OV-3, PC-3, T-47D as well as HEK-293 and PBMC (50 µM in all cell lines).

Supporting Information for Chapter II

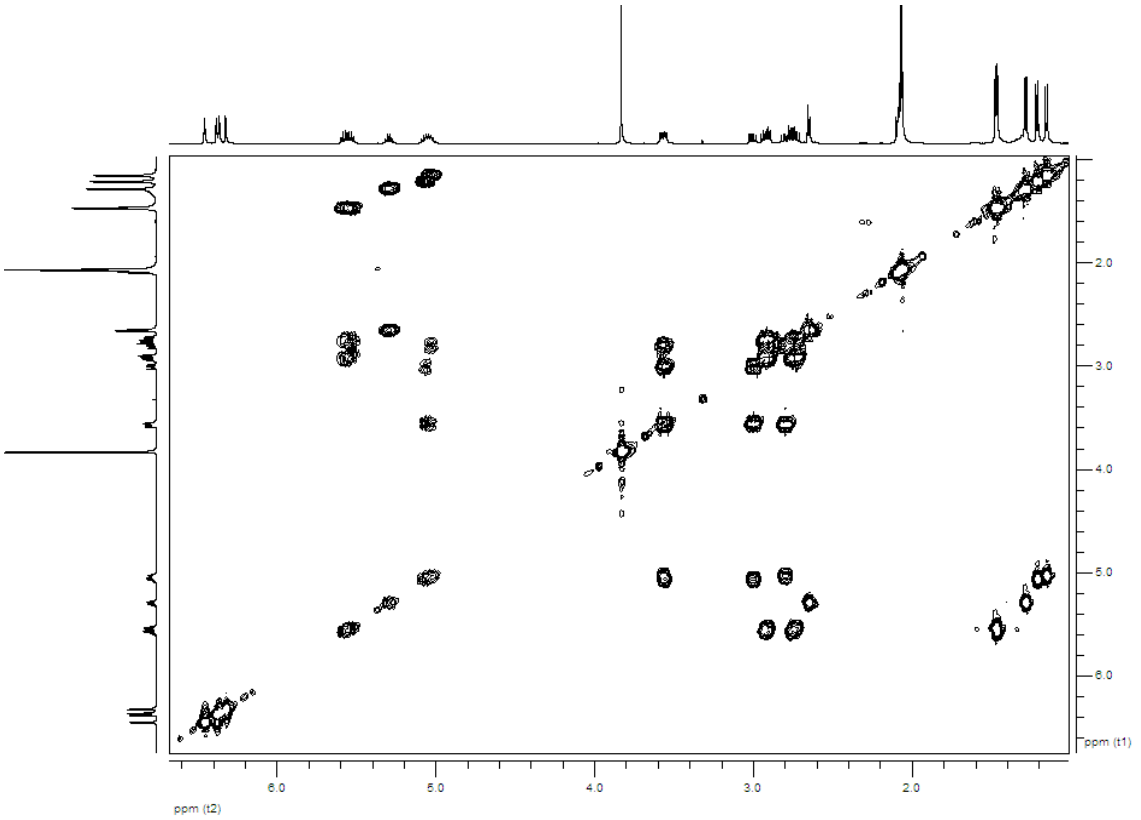
AII.1. ^{1}H NMR (500 MHz, acetone-d_6) spectrum of calcaride A (**6**).



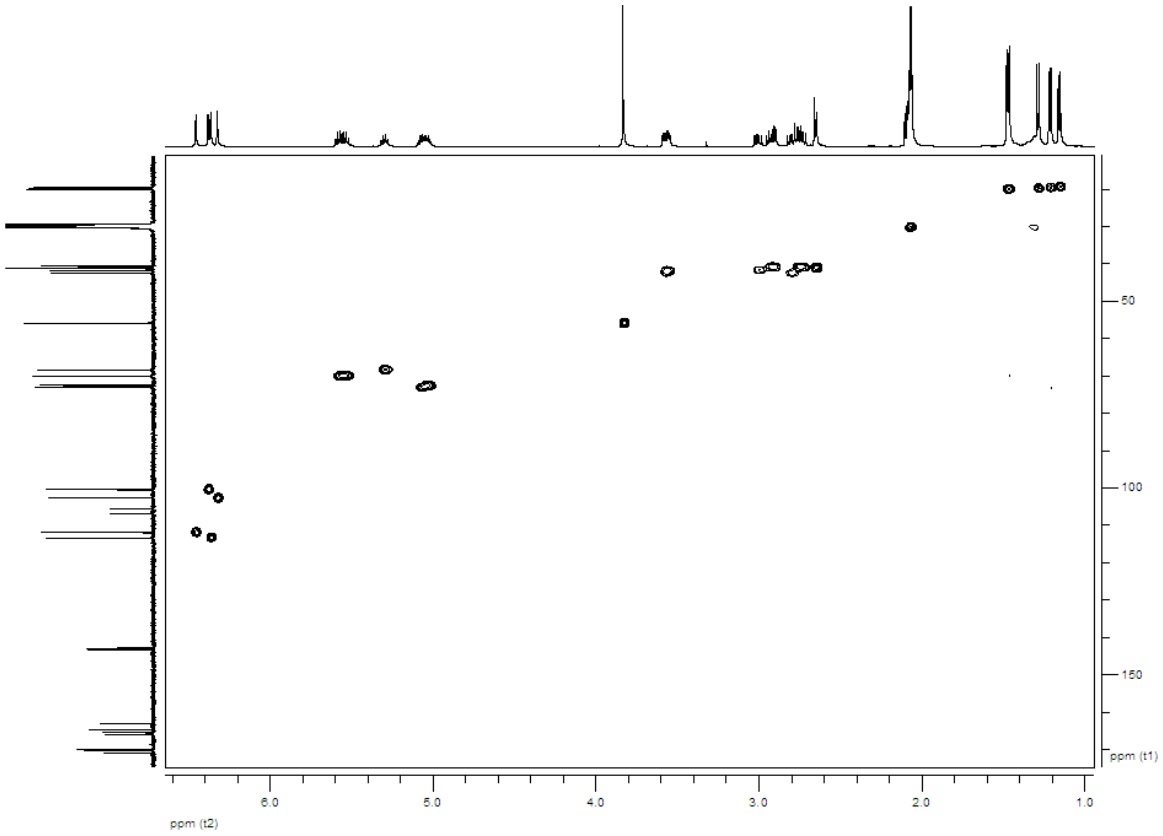
AII.2. ^{13}C NMR (500 MHz, acetone-d_6) spectrum of calcaride A (**6**).



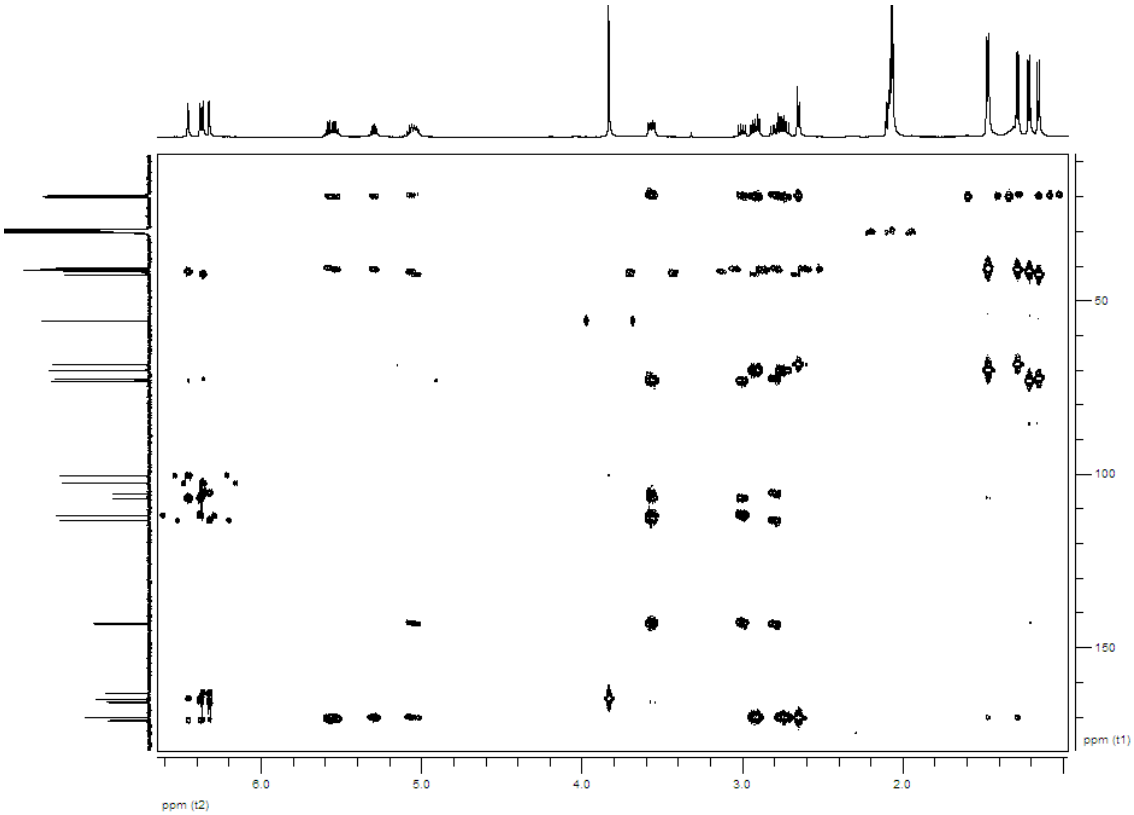
AII.3. COSY (500 MHz, acetone-d_6) spectrum of calcaride A (**6**).



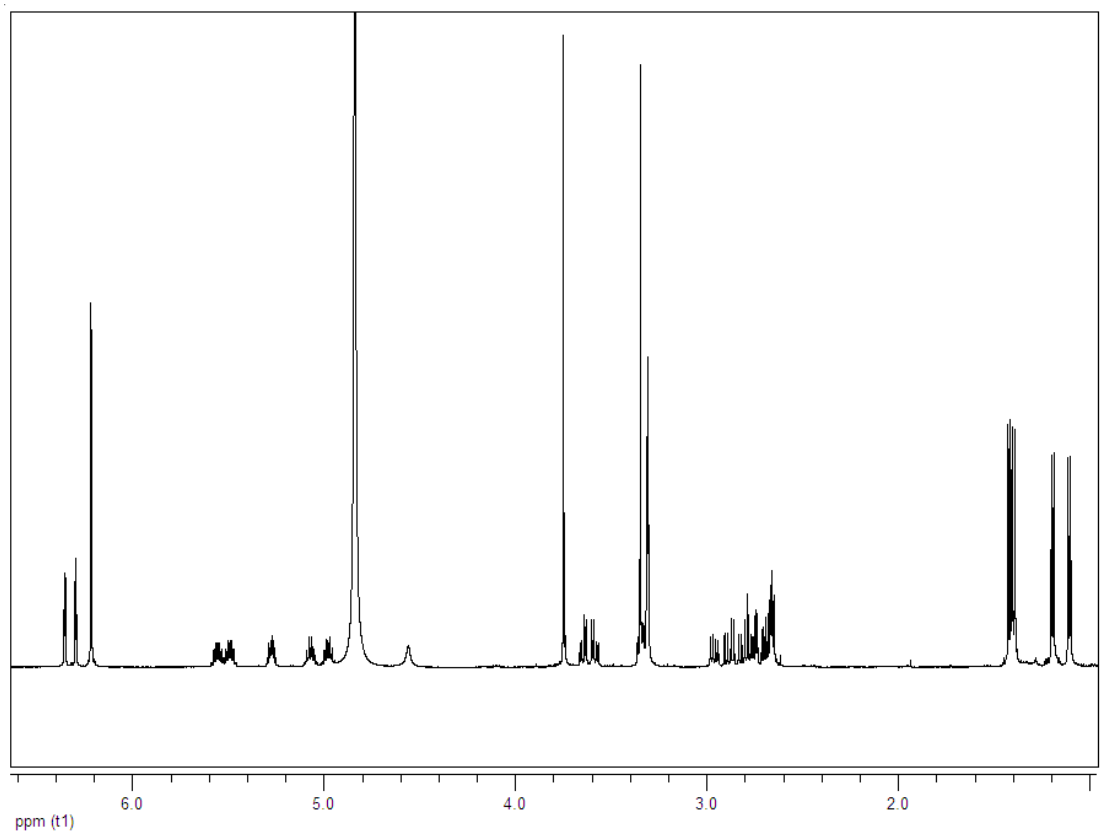
AII.4. HSQC (500 MHz, acetone-d_6) spectrum of calcaride A (**6**).



AII.5. HMBC (500 MHz, acetone-d_6) spectrum of calcaride A (**6**).



AII.6. ^{1}H NMR (500 MHz, methanol-d_4) spectrum of calcaride B (**7**).

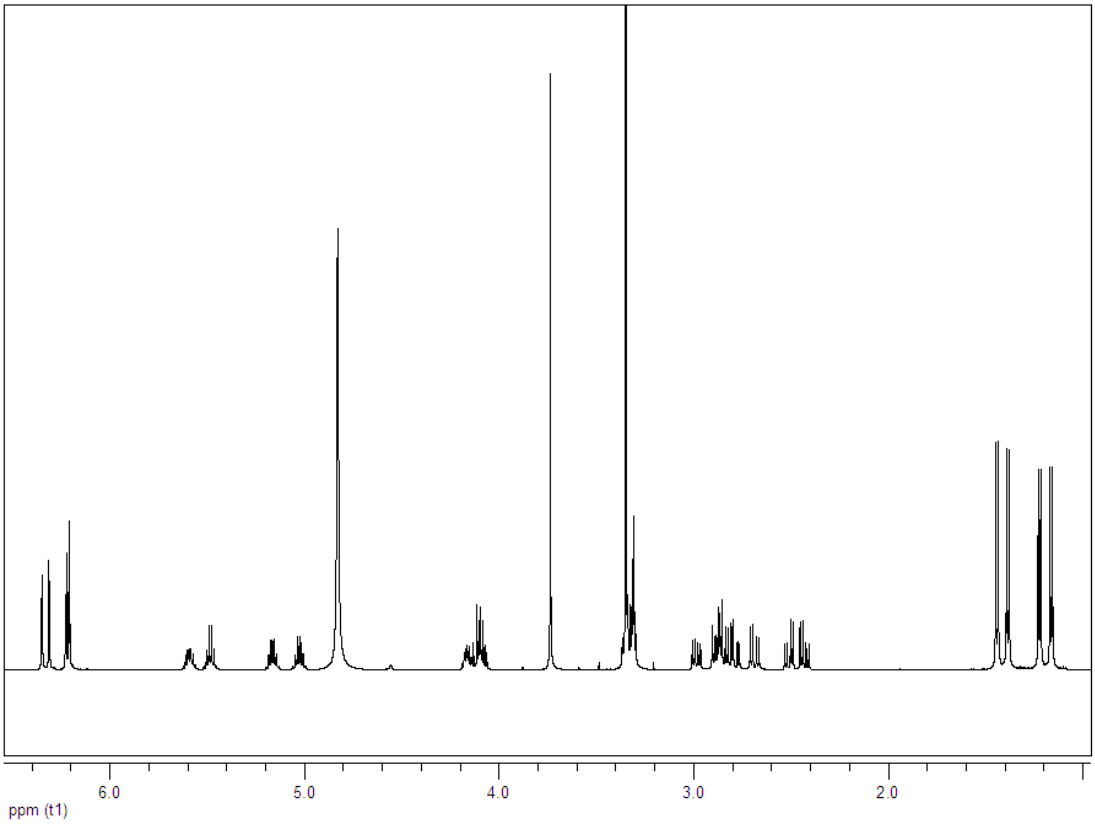


AII.7. NMR spectroscopic data (500 MHz, methanol-d_4) of calcaride B (**7**).

Position	δ_C, Type	δ_H, Mult. (*J* in Hz)	COSY	HMBC
1	171.5, C[a]			
2a	41.0, CH_2	2.88, dd (7.7, 16.6)	2b, 3	1, 3, 4, 5
2b		2.73, d (5.3, 16.6)	2a, 3	1, 3, 4, 5
3	70.2, CH	5.56, m	2a, 2b, 4	2, 4, 5
4	20.2, CH_3	1.42, d (6.3)	3	2, 3
5	171.13, C			
6	108.7, C			
7	164.6, C			
8	100.7, CH	6.30, d (2.6)	10	5, 6, 7, 10
9	164.9, C			
OCH_3	55.8, CH_3	3.75, s		9
10	111.5, CH	6.36, d (2.6)	8	5, 6, 8, 9, 12
11	142.6, C			
12a	41.8, CH_2	3.34[b]	12b, 13	6, 10, 11, 13, 14
12b		2.96, dd (6.4, 13.8)	12a, 13	6, 10, 11, 13, 14
13	73.6, CH	5.07, m	12a, 12b, 14	11, 12, 14, 15
14	20.0, CH_3	1.20, d (6.2)	13	12, 13
15	171.37, C[a]			
16a	41.6, CH_2	2.81, dd (7.6, 15.8)	16b, 17	15, 17, 18
16b		2.67, dd (5.7, 15.8)	16a, 17	15, 17, 18
17	70.4, CH	5.49, m	16a, 16b, 18	16, 18, 19
18	20.2, CH_3	1.40, d (6.4)	17	16, 17
19	171.07, C			
20	106.7, C			
21	165.4, C			
22	102.8, CH	6.22, s		19, 20, 21, 23, 24, 26
23	163.5, C			
24	113.3, CH	6.22, s		19, 20, 21, 22, 23, 26, 27
25	143.2, C			
26a	42.3, CH_2	3.32[b]	26b, 27	20, 24, 25, 27, 28
26b		2.77, dd (8.1, 13.3)	26a, 27	20, 24, 25, 27, 28
27	73.5, CH	4.98, m	26a, 26b, 28	25, 26, 29
28	19.5, CH_3	1.11, d (6.2)	27	26, 27
29	171.43, C[a]			
30a	36.7, CH_2	2.68, dd (5.1, 16.7)	30b, 31	29, 31, 32
30b		2.64, dd (8.1, 16.7)	30a, 31	29, 31, 32
31	72.7, CH	5.28, m	30a, 30b, 32a, 32b	1, 29, 30
32a	63.6, CH_2	3.65, dd (4.5, 11.9)	31, 32b	30, 31
32b		3.58, dd (5.1, 11.9)	31, 32a	30, 31

[a]Assignments of C-1, C-15 and C-29 are interchangeable. [b]Signal partially obscured and deduced from the HMBC NMR.

AII.8. ^{1}H NMR (500 MHz, methanol-d_4) spectrum of calcaride C (**8**).

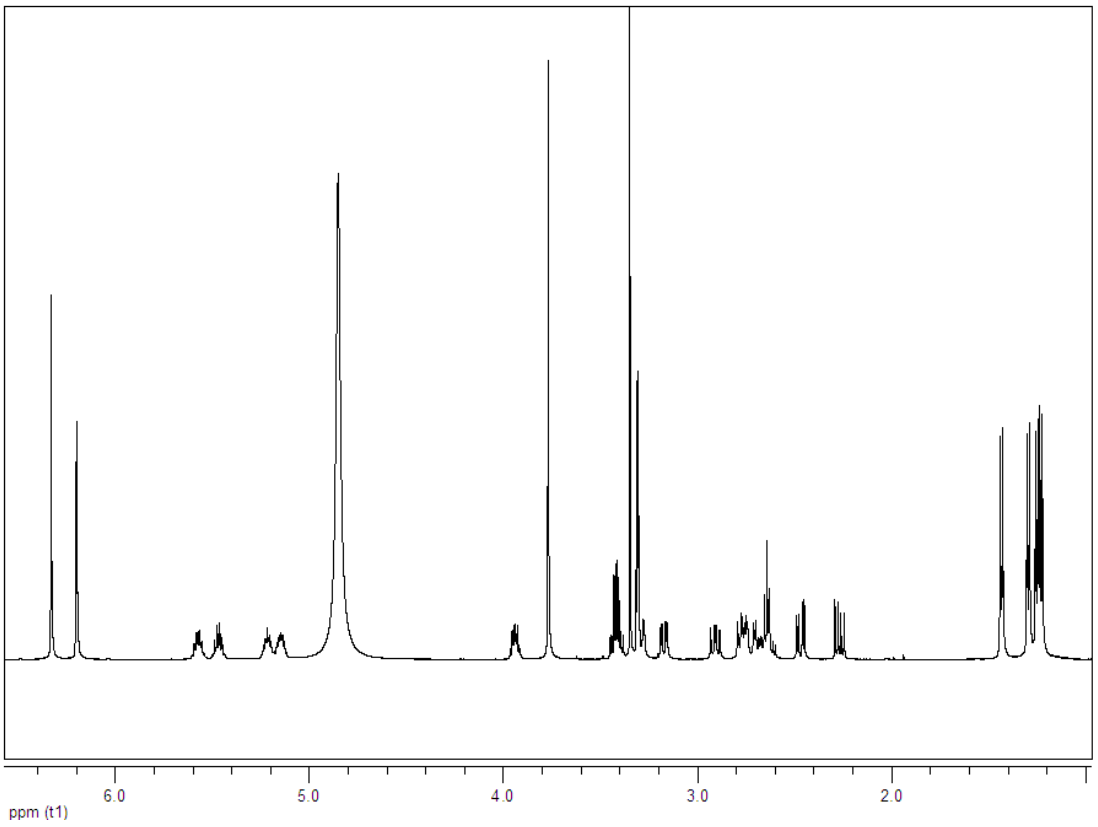


AII.9. NMR spectroscopic data (500 MHz, methanol-d_4) of calcaride C (**8**).

Position	δ_C, Type	δ_H, Mult. (*J* in Hz)	COSY	HMBC
1	171.6, C			
2a	41.2, CH_2	2.878, dd (8.1, 16.2)	2b, 3	1, 3, 4
2b		2.79, dd (4.8, 16.2)	2a, 3	1, 3, 4
3	70.5, CH	5.59, m	2a, 2b, 4	2, 4, 5
4	20.2, CH_3	1.44, d (6.4)	3	2, 3
5	171.53, C[a]			
6	108.2, C			
7	165.08, C[a]			
8	100.7, CH	6.31, d (2.6)	10	5, 6, 7, 9, 10, 12
9	165.0, C[a]			
OCH_3[b]	55.8, CH_3	3.74, s		9 (or 7)
10	111.3, CH	6.35, d (2.6)	8	5, 6, 7, 8, 9, 12
11	142.7, C			
12a	41.7, CH_2	3.35[c]	12b, 13	6, 10, 11, 13, 14
12b		2.99, dd (6.6, 14.0)	12a, 13	6, 10, 11, 13, 14
13	73.2, CH	5.16, m	12a, 12b, 14	11, 12, 14, 15
14	20.0, CH_3	1.22, d (6.2)	13	12, 13
15	171.3, C[a]			
16a	41.6, CH_2[a]	2.85, dd (6.6, 16.0)	16b, 17	15, 17, 18
16b		2.69, dd (6.6, 16.0)	16a, 17	15, 17, 18
17	70.3, CH	5.48, m	16a, 16b, 18	16, 18, 19
18	20.1, CH_3	1.39, d (6.3)	17	16, 17
19	171.48, C[a]			
20	107.3, C			
21	165.14,C[a]			
22	102.7, CH	6.21, d (2.5)		19, 20, 21, 23, 24, 26
23	163.4, C			
24	112.6, CH	6.23, d (2.5)		19, 20, 21, 22, 23, 26
25	143.1, C			
26a	41.5, CH_2[a]	3.31[c]	26b, 27	20, 24, 25, 27, 28
26b		2.885, dd (6.9, 13.4)	26a, 27	20, 24, 25, 27, 28
27	73.6, CH	5.03, m	26a, 26b, 28	25, 26, 28, 29
28	19.8, CH_3	1.16, d (6.2)	27	26, 27
29	171.9, C			
30a	40.4, CH_2	2.51, dd (6.0, 15.3)	30b, 31	29, 31, 32
30b		2.43, dd (7.2, 15.3)	30a, 31	29, 31, 32
31	67.1, CH	4.17, m	30a, 30b, 32a, 32b	29, 30, 32
32a	68.5, CH_2	4.12, dd (4.8, 11.1)	31, 32b	1, 30, 31
32b		4.08, dd (5.5, 11.1)	31, 32a	1, 30, 31

[a]Assignments of C-5, C-15 and C-19, C-7 and C-21, C-7 and C-9, C-16 and C-26, respectively are interchangeable. [b]Position of the methoxy group could not unambiguously be determined. In analogy to the other calcarides it was assumed to be linked to C-9. [c]Signal partially obscured.

AII.10. ^{1}H NMR (500 MHz, methanol-d_4) spectrum of calcaride D (**9**).

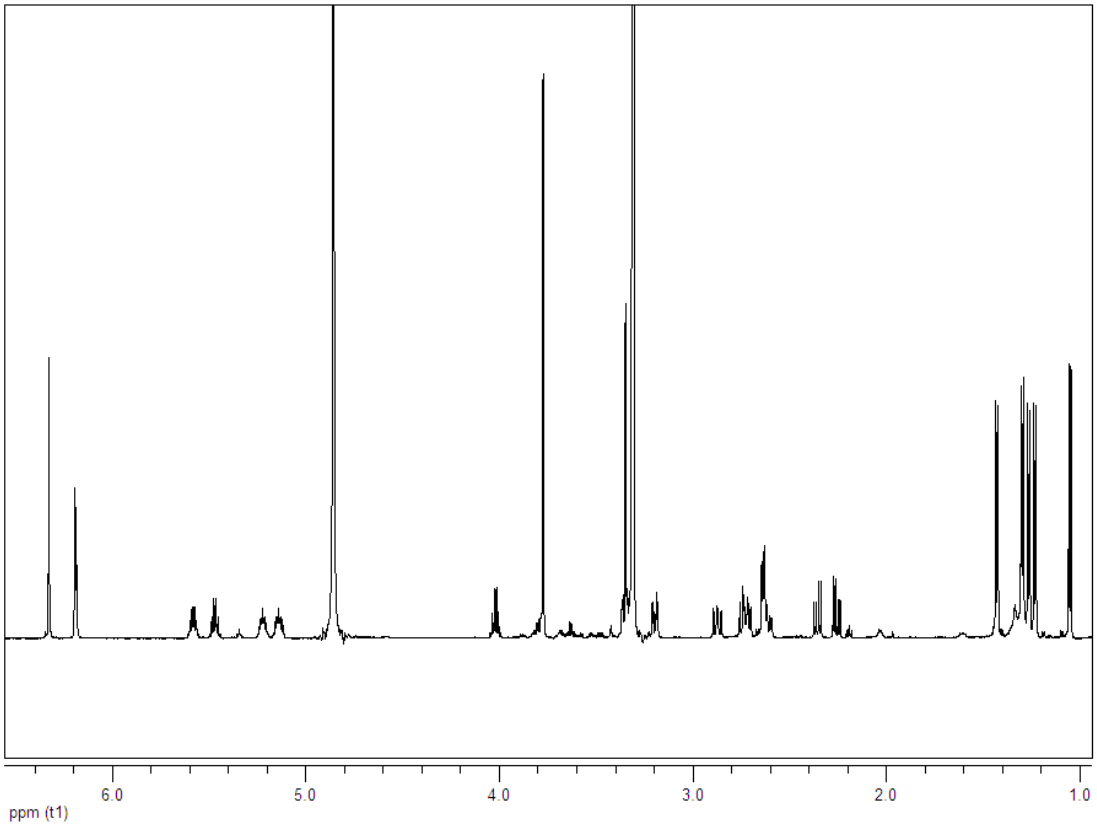


AII.11. NMR spectroscopic data (500 MHz, methanol-d_4) of calcaride D (**9**).

Position	**δ_C, Type**	**δ_H, Mult. (*J* in Hz)**	**COSY**	**HMBC**
1	173.9, C[a]			
2a	41.8, CH_2	2.767, dd (7.5, 15.8)	2b, 3	1, 3, 4
2b		2.69, dd (5.3, 15.8)	2a, 3	1, 3, 4
3	70.8, CH	5.57, m	2a, 2b, 4	1, 2, 4, 5
4	20.1, CH_3	1.43, d (6.4)	3	2, 3
5	171.5, C[b]			
6	107.2, C			
7	166.0, C[b]			
8	100.8, CH	6.33, s		5, 6, 7, 9, 10
9	165.0, C[b]			
OCH_3	55.9, CH_3	3.77, s		9
10	112.8, CH	6.33, s		5, 6, 7, 8, 9, 12, 13
11	143.2, C			
12a	43.2, CH_2	3.29[c]	12b, 13	6, 10, 11, 13, 14
12b		2.91, dd (9.7, 13.7)	12a, 13	6, 10, 11, 13, 14
13	73.1, CH	5.22, m	12a, 12b, 14	11, 12, 14, 15
14	20.52, CH_3	1.25, d (6.2)	13	11, 12, 13
15	171.2, C			
16a	41.8, CH_2	2.66, dd (7.2, 15.7)	16b, 17	15, 17, 18
16b		2.62, dd (5.8, 15.7)	16a, 17	15, 17, 18
17	70.0, CH	5.47, m	16a, 16b, 18	16, 18, 19
18	19.9, CH_3	1.30, d (6.4)	17	15, 16, 17
19	171.6, C[b]			
20	105.8, C			
21	166.2, C			
22	102.7, CH	6.203, d (2.6)		20, 21, 23, 24
23	163.5, C			
24	113.8, CH	6.197, d (2.6)		19, 20, 21, 22, 23, 26, 27
25	143.7, C			
26a	43.5, CH_2	3.17, dd (4.0, 13.4)	26b, 27	20, 24, 25, 27, 28
26b		2.772, dd (9.2, 13.4)	26a, 27	20, 24, 25, 27, 28
27	72.7, CH	5.15, m	26a, 26b, 28	25, 26, 28, 29
28	20.49, CH_3	1.23, d (6.2)	27	25, 26, 27
29	172.7, C			
30a	39.9, CH_2	2.47, dd (5.2, 15.4)	30b, 31	29, 31, 32
30b		2.27, dd (7.9, 15.4)	30a, 31	29, 31, 32
31	69.9, CH	3.94, m	30a, 30b, 32a, 32b	29, 30, 32
32a	66.4, CH_2	3.44, dd (4.9, 11.2)	31, 32b	30, 31
32b		3.40, dd (5.7, 11.2)	31, 32a	30, 31

[a]Signal deduced from the HMBC NMR spectrum. [b]Assignments of C-5 and C-19, C-7 and C-9, respectively are interchangeable. [c]Signal partially obscured and deduced from the HSQC NMR spectrum.

AII.12. ^{1}H NMR (600 MHz, methanol-d_4) spectrum of calcaride E (**10**).



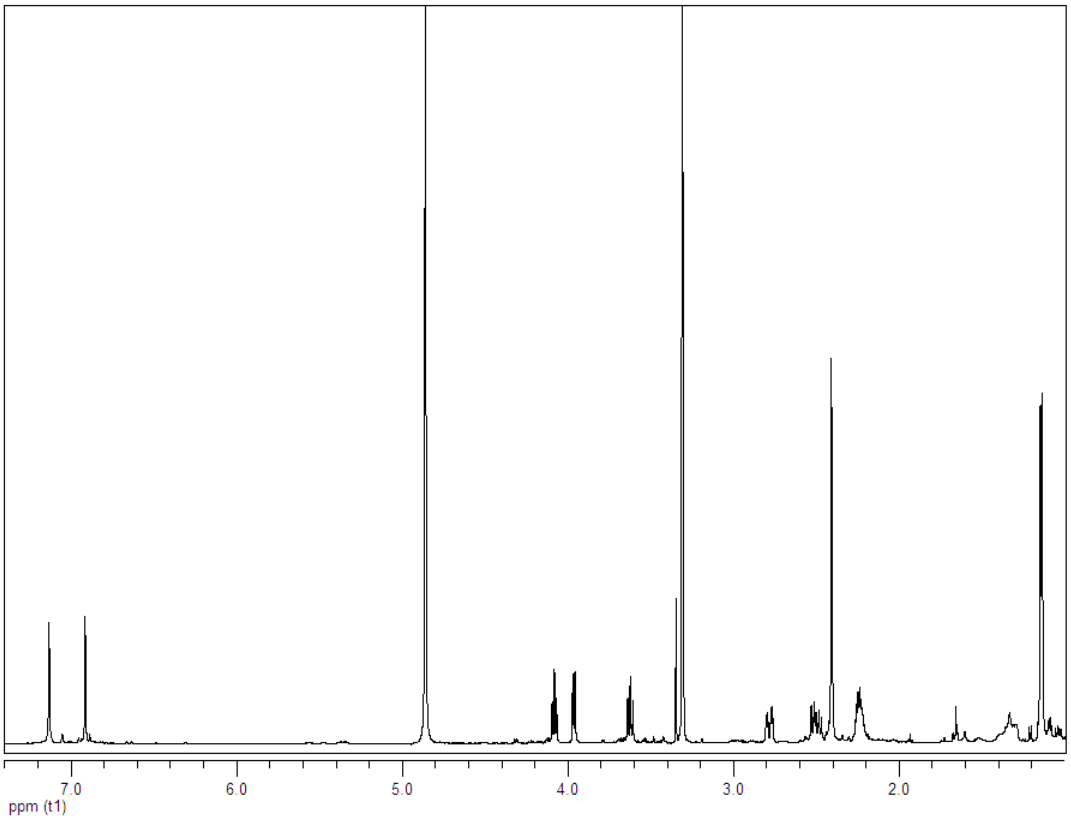
AII.13. NMR spectroscopic data (600 MHz, methanol-d_4) of calcaride E (**10**).

Position	δ_C, Type	δ_H, Mult. (*J* in Hz)	COSY	HMBC
1	175.9, C[a]			
2a	43.3, CH_2	2.72, dd (7.8, 15.4)	2b, 3	1, 3, 4
2b		2.61, dd (5.9, 15.4)[b]	2a, 3	1, 3, 4
3	71.5, CH	5.58, m	2a, 2b, 4	1, 2, 4, 5
4	20.3, CH_3	1.43, d (6.3)	3	2, 3
5	171.56, C[c]			
6	107.3, C			
7	166.0, C[c]			
8	100.8, CH	6.33, s		5, 6, 7, 9, 10
9	165.0, C[c]			
OCH_3	55.9, CH_3	3.77, s		9
10	112.8, CH	6.33, s		5, 6, 7, 8, 9, 12, 13
11	143.3, C			
12a	43.4, CH_2	3.35, dd (3.5, 13.6)[b]	12b, 13	6, 10, 11, 13
12b		2.87, dd (9.8, 13.6)	12a, 13	6, 10, 11, 13, 14
13	73.2, CH	5.22, m	12a, 12b, 14	11, 15
14	20.6, CH_3	1.26, d (6.3)	13	11, 12, 13
15	171.2, C			
16a	41.8, CH_2	2.65, dd (7.2, 15.6)	16b, 17	15, 17, 18
16b		2.62, dd (6.0, 15.6)	16a, 17	15, 17, 18
17	70.0, CH	5.47, m	16a, 16b, 18	15, 16, 18, 19
18	19.9, CH_3	1.30, d (6.3)	17	15, 16, 17
19	171.62, C[c]			
20	105.7, C			
21	166.3, C			
22	102.7, CH	6.19, d (2.5)		19, 21, 20, 23, 24
23	163.6, C			
24	113.9, CH	6.18, d (2.5)		20, 21, 22, 23, 26
25	143.7, C			
26a	43.6, CH_2	3.20, dd (3.8, 13.5)	26b, 27	20, 24, 25, 27, 28
26b		2.74, dd (9.5, 13.5)	26a, 27	20, 24, 25, 27, 28
27	72.6, CH	5.14, m	26a, 26b, 28	25, 29
28	20.6, CH_3	1.23, d (6.2)	27	25, 26, 27
29	172.5, C			
30a	45.1, CH_2	2.36, dd (6.9, 14.7)	30b, 31	29, 31, 32
30b		2.26, dd (6.5, 14.7)	30a, 31	29, 31, 32
31	65.4, CH	4.02, m	30a, 30b, 32	29, 30, 32
32	23.0, CH_3	1.05, d (6.2)	31	30, 31

[a]Signal deduced from the HMBC NMR spectrum. [b]Signal partially obscured. [c]Assignments of C-5 and C-19, C-7 and C-9, respectively are interchangeable.

Supporting Information for Chapter III

AIII.1. ^{1}H NMR (600 MHz, methanol-d_4) spectrum of malettinin E (**4**).



AIII.2. ^{13}C NMR (150 MHz, methanol-d_4) spectrum of malettinin E (**4**).